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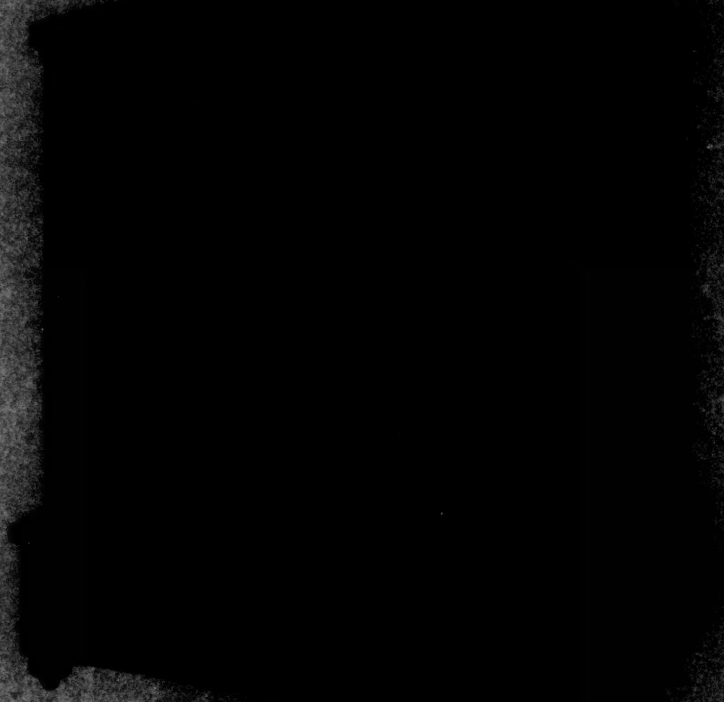

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THE QUANTITATIVE DETERMINATION OF THE TOTAL PROTEIN AND NON-PROTEIN SUBSTANCES OF MUSCLE.

IMPROVED TECHNIQUE.

By N. W. JANNEY.

(*From the Chemical Laboratory of the Montefiore Home and Hospital for Chronic Invalids, New York.*)

(Received for publication, April 10, 1916.)

A method for the quantitative determination of the protein and non-protein muscle substances was described in a previous communication.[1] The principle involved was coagulation of the protein substances with aid of acetic acid and sodium chloride, followed by separation from the non-protein material through extracting with water, alcohol, and ether. It has, however, been ascertained as a result of added experience that the technique can be considerably improved. Particularly when the muscle was not fresh it was observed that a sample could rarely be met with in which the protein failed to be rendered entirely insoluble by acetic acid and saline, even when the coagulation was carried out with the greatest pains. On investigation, the loss of protein arising from this cause usually proved inconsiderable, but in the three highest instances it was found to lie between 1.0 and 3.5 per cent. The subsequent filtering and washing was tediously delayed under these circumstances.

Owing to the need of a reliable method for the determination of the meat proteins, efforts were made to develop a more satisfactory procedure. No departure was made from the principle of direct determination of the protein substance inasmuch as a complete separation of this material from the non-proteins is also essential for an accurate indirect determination of the protein by means of nitrogen analyses. Consequently, in seeking for a more

[1] Janney, N. W., and Csonka, F. A., *J. Biol. Chem.*, 1915, xxii, 195.

reliable means of protein precipitation with a view to its direct determination, it was preferred to avoid the use of metallic salts employed in a number of the older procedures. In using such agents there is danger of producing difficultly soluble nitrogenous precipitates other than protein which represent a source of error, whether the protein is determined *per se* or by calculating the amount present from the nitrogen content.

Alcohol was found to present decided advantages as a protein precipitant. All varieties of muscle examined were found to undergo ready and practically complete coagulation in hot alcohol without the observation of special precautions. Most of the fatty substances of muscle go into solution at the same time. This greatly facilitates the subsequent filtration and extraction and represents an important advantage over an aqueous medium for rendering the muscle proteins insoluble. Thus we have found that the average length of time necessary for coagulation and extraction with alcohol and water as later detailed is 15 minutes. A modified method was therefore developed on this basis which is described at the end of the article. This procedure, though not expeditious, presents little technical difficulty and leads to satisfactory results.

In order to determine the efficacy of alcohol as a protein precipitant applied to muscle, considerable pains were devoted to examining the extracts obtained by employment of the new analytical procedure for protein. It was thought advisable in these controls to rely mainly upon the use of protein precipitants of recognized value. Trichloroacetic acid has recently been used in the removal of blood proteins by Greenwald[2] whose experiments have demonstrated its reliability for this purpose. It was therefore utilized as follows with quantitative precautions. Meat was extracted according to the detailed description of the method as given below. The combined alcoholic and aqueous extracts so obtained were made neutral and concentrated under greatly diminished pressure at 40° till the liquid attained a volume of 40 to 60 cc. It was then transferred to a continuous extraction apparatus and the fatty substances were completely removed by the prolonged action of ether. The liquid was then precipitated with nine

[2] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

volumes of 2.5 per cent acetic acid, according to Greenwald's technique. The precipitate was collected on a weighed filter, washed with water containing trichloroacetic acid, dried, and the weight of the recovered protein ascertained. For the sake of comparison, in duplicate analyses the concentrated liquid after the ether extraction was precipitated with three volumes of 95 per cent alcohol, washed, dried, and the protein so obtained weighed. 10 gm. of muscle were used in these control analyses throughout. The results are collected as follows.

TABLE I.

Species.	Protein recovered from filtrate by	
	Trichloroacetic acid.	Alcohol.
	gm.	*gm.*
Ox I*	0.0069	
" II	0.0065	
Ox I	0.0021	
" II		0.0270
Fish (halibut) I	0.0252	
" " II		0.0469

* Duplicate analyses thus designated.

It is evident that but a very small fraction of the muscle and blood proteins contained in muscle escapes precipitation when alcohol is used for coagulation. A much greater amount of material of probable protein nature could be recovered from the extracts by alcohol than by trichloroacetic acid. In the series of controls appearing in Table II, alcohol was therefore used to obtain the lost protein. In this case the combined alcoholic and aqueous extracts were concentrated as previously mentioned, three volumes of 95 per cent alcohol added, after standing the precipitate collected on a weighed filter, thoroughly washed with 95 per cent alcohol, which also removed all remaining aqueous extractives, then extracted in a continuous extractor with absolute alcohol followed by ether, dried, and weighed. The material so obtained is regarded as protein[3] and is noted in the table.

Ash was found to be the only impurity present in estimable amounts in the protein obtained by the method as described.

[3] It is probable that the material of protein nature thus recovered by reprecipitation with alcohol represents albumoses and peptones. As the

TABLE II.

Control Muscle Analyses.

Species.	Muscle for analysis.	Protein found.		Protein recovered from extracts.	Error due to	
					Protein lost in extracts.	Ash in protein found.
	gm.	*gm.*	*per cent*	*mg.*	*per cent*	*per cent*
Rabbit	10.0053	1.6250	16.2			+0.055
Cat I*	9.9945	1.7548	17.6	38.7	−0.39	+0.09
" II	9.9860	1.7820	17.8	33.8	−0.34	
Dog I	10.0030	1.729	17.3	29.4	−0.29	+0.08
" II	9.9995	1.722	17.2	29.8	−0.30	
Fish (halibut)	9.9935	1.6705	16.7	16.4	−0.16	+0.05
Ox	10.0000	1.7230	17.2	24.7	−0.25	
"	10.0000	1.4060	14.1	11.2	−0.05	+0.01
" I	10.0109	1.6995	17.0	6.3	−0.06	+0.07
" II	10.0143	1.7008	17.0	8.0	−0.08	
Man I**	10.0000	1.2010	12.0	1.1	−0.01	+0.04
" II	10.0000	1.2035	12.0	4.0	−0.04	
"	9.9930	1.7417	17.4	2.4	−0.02	+0.05
" **	10.0095	1.4595	14.6	7.5	−0.07	+0.02
" I	10.0175	1.6971	16.9	10.2	−0.10	+0.07
" II	9.9815	1.6548	16.6	18.3	−0.20	

*Duplicate analyses thus designated.

**From emaciated subject.

The extent to which the percentage values of protein found were influenced by this source of error has been calculated from the results of ash determinations made from this material (Table II). This error is on the positive side and thus serves to counterbalance,

latter are not precipitated by trichloroacetic acid, the difference in the results obtained by these protein precipitants may be ascribed to this cause. Evidently albumoses and peptones are present in very small quantities in fresh meat.

at least partially, the loss of material of protein nature in the extracts described above.

The protein preparations obtained by aid of the new analytical procedure were, as hitherto, also tested for the presence of carbohydrates. Carbohydrates could only be detected with aid of the sensitive Molisch reaction in about half of the cases examined. As the slightest contamination with filter paper leads to a positive result when this delicate test is employed, such an eventuality must be taken into account in judging these results.

It had previously been found impossible to remove the fat entirely when the protein was coagulated in an aqueous medium. The presence of fat could not, however, be detected in the protein residuum obtained by the aid of the present modified technique. The method employed to ascertain this fact was that of Gephart and Csonka[4] with employment of 0.01 N solutions and control of all reagents. *The conclusion may therefore be drawn that, contrary to the generally accepted view, it is possible to free muscle completely of fatty substances by extraction with use of the analytical procedure here described.* The finding of a residuum of fat in muscle extracted even for weeks has been variously reported in the past. This result is due to the methods used. Usually air- or vacuum-dried muscle was extracted with ether. Such material is so inspissated that it is extremely resistant to solvents. Inadequacies in older methods of fat determinations probably represent an additional factor contributing to such results.

The preparations of protein obtained on completion of the analyses are very light in color. It seems scarcely possible that this degree of pigmentation can represent a very appreciable cause of inexactness.

Another possible source of error remains to be discussed, the effect of extraction and the necessary mechanical manipulation on the filter paper weighed with the protein. Two Schleicher and Schull No. 597 papers, 12.5 cm. in diameter, those commonly used by us in this method, were dried to constant weight and then treated precisely as called for in carrying out the analytical procedure except that the muscle was omitted. The weight lost was 0.6 and 0.3 mg. respectively. The error from this source is therefore inconsequential.

[4] Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

In the previous study considerable time had already been devoted to determining the duration of the Soxhlet extraction required for the complete removal of the fatty substances. In the hope, however, that the preliminary treatment with alcohol as now employed would shorten this period, suitable control experiments were carried out in establishing the accuracy of the present modification. The amount of solids present in the extract was ascertained at intervals, the same precautions being observed as previously.[1] It was found that all but a few mg. of alcohol-soluble material is removed from the protein within 6 hours. This residuum of fatty substances is, however, extracted but slowly, so that a total of 18 hours as compared to 24 previously is found necessary to insure complete extraction. In these controls the ordinary type of Soxhlet apparatus and an electric hot plate were employed. As the length of time required for the complete removal of all extractives varies to some extent according to the amount of extractives, especially fat, present, also with the individual apparatus and precise temperature employed, it is advisable in very exact work to ascertain this point in advance by control of the extract as indicated. An 18 hour extraction as described in detail below represents an entirely safe margin, even when considerable fat is present in the muscle.

We have found that the use of ether is attended by no obvious advantage over alcohol. All ether-soluble substances are removed by the alcohol. This was established by a further series of control experiments which are omitted for the sake of brevity.

Accuracy of the Modified Method.—In summarizing the various experiments made to this end, it is believed that consideration has been given to all apparent sources of error which can be controlled. The protein values found are influenced to the average extent of -0.15 by loss of material of protein nature in the extracts, $+0.05$ by the ash, and ± 0.15 per cent in duplicate analyses. The method properly carried out may therefore be regarded as accompanied by a total average error probably not exceeding a few tenths of 1 per cent.

Improved Method in Detail.

The fresh muscle is freed from all adherent fat and connective tissue, passed through a meat grinder, and thoroughly mixed.

About 10 gm. are weighed by difference into a beaker from a weighing glass provided with a ground glass lid. 50 cc. of 95 per cent alcohol are added and the contents of the beaker heated with stirring until the alcohol boils. The liquid is then decanted through an ordinary round filter of 12.5 cm. diameter, which has previously been extracted with alcohol and ether, dried, and weighed. This treatment of the protein with alcohol is once repeated. The coagulated muscle is next extracted in a similar manner with 400 cc. of boiling water in four portions, and then brought quantitatively on the filter. The filter is now carefully folded about the protein material, which is gently inserted into an extraction hull and extracted 3 hours in an ordinary Soxhlet apparatus with 95 per cent alcohol. The 95 per cent alcohol is then replaced by absolute alcohol and the extraction continued for a period of 15 hours. Care must be taken that the filter projects beyond the upper level attainable by the solvent, which must completely surround the protein. After completion of the extraction the filter with the pure protein is removed from the apparatus, dried to constant weight at 105° in a weighing glass provided with a ground glass lid, and the previously ascertained weight of the filter paper deducted.

As 10 gm. muscle samples have been used in the control analyses, this amount is adhered to in the above description. In view of the small error found, the employment of 5 gm. of muscle, with correspondingly smaller amounts of alcohol for coagulation and water for extraction, is permissible. It is indeed an advantage, because the time required for drying the protein and probably also for its Soxhlet extraction is somewhat diminished.

The *non-protein substances* are determined when required, by deducting the per cent of protein found from that of the total solids. which are estimated as described in the earlier communication.[1]

With the help of this method, we have detected a considerable error in the usual modes of estimation of the amount of protein present in muscle. This subject is treated in the note immediately following the present article.[5]

[5] Attention has also been called to these studies in a recent paper read before the Society for Experimental Biology and Medicine (*Proc.*, 1916, xiii, 83).

THE PROTEIN CONTENT OF MUSCLE.

By N. W. JANNEY.

(From the Chemical Laboratory of the Montefiore Home and Hospital for Chronic Invalids, New York.)

(Received for publication, April 10, 1916.)

An exact knowledge of the composition of animal muscle is of considerable importance for the science of nutrition. Though the proteins represent the most important muscle constituents, the usual means employed to determine the amount of these substances present remain rather inaccurate. Thus the amount of protein in muscle is still most commonly estimated by multiplying the total nitrogen by the classical protein factor 6.25. This mode of calculation is used in most of the standard food analyses tables, such as published by König,[1] Atwater and Bryant,[2] Atwater,[3] and Lusk.[4] On the protein values so obtained are based various metabolic data including calculations of the caloric values. Although this method of estimating muscle protein is generally known to be a faulty one, its inadequacies may still be alluded to in the hope of lessening its present extensive employment. The protein factor 6.25 is based on acceptance of 16.00 per cent as the nitrogen content of muscle proteins. Although many of the older nitrogen analyses of muscle proteins do give values averaging about 16.00 per cent, the muscle protein preparations then analyzed were of very dubious purity. More recently Osborne and his coworkers have reported analyses of carefully prepared animal muscle proteins which are considerably higher than 16.00 per cent. Similar analytical results have also been obtained in the writer's laboratory for pure muscle proteins. The nitrogen content was found to be

[1] König, J., Chemie der Menschlichen Nahrungs- und Genussmittel, Berlin, 4th edition, 1910, iii, 252.

[2] Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Bull. 28*, 1906.

[3] Atwater, W. O., *U. S. Dept. Agric., Farmer's Bull. 142*, 1906.

[4] Lusk, G., The Elements of the Science of Nutrition, Philadelphia, 2nd edition, 1909.

rather constant for various species of higher animals, ranging from 16.2 to 16.7 per cent in many analyses. In view of this work the protein factor 6.25 can no longer be considered accurate. Moreover, a very considerable error is introduced by using the total nitrogen of muscle in this mode of calculation of the protein, for our analyses have shown that about 13 per cent of the total nitrogen is combined in non-protein substances in muscle. The amount of protein obtained is greatly increased in consequence.

A further source of confusion and error is the loose application of the term "protein" to include practically all protein and "nitrogenous non-protein substances" found in animal muscle. Thus Atwater and Bryant, who adopt this nomenclature, calculate the number of calories obtainable from the proteins of meats from protein in this sense. As extractive substances of non-protein nature are here reckoned in, the result must be inaccuracy in the number of calories ascribed to the meat protein.

Another much used indirect method for estimating the amount of muscle protein is that "by difference." According to this plan the total water-free substance is ascertained; and from this value the solid material found in the ether extract of the dried muscle, together with the ash, is subtracted. The result is designated as protein and varies but slightly from that obtained by multiplying the total muscle nitrogen by the factor 6.25. This correspondence is, however, no criterion of accuracy, for nearly the same sources of error are present in both procedures. The "protein" as thus ascertained from anhydrous muscle material includes also the non-protein extractives aside from those soluble in ether. In the article immediately preceding mention has also been made of the fact that it is very difficult to free dried muscle entirely of fatty substances by means of ether extraction. Any residual amount of fat thus remaining in the extracted protein would serve to increase the protein content obtained by this mode of calculation. This presents an additional source of inaccuracy. Lastly the carbohydrate content of muscle is likewise reckoned as protein by this procedure. A further small but appreciable error is thus introduced.

To enter into a discussion of various other methods proposed for the estimation of muscle proteins is scarcely advisable in view of the fact that none have come into very general use. The chief

reason for this is that these procedures are of questionable accuracy in nearly all cases.

These considerations have led the writer to devise a new method for the determination of the muscle proteins which permits of a rather exact determination of these substances. The improved technique of this method is described in the preceding article.

In the table are arranged for comparison protein values taken from Atwater and Bryant's food analyses obtained by multiplication of the total muscle nitrogen by the factor 6.25, as well as "by difference," as described above. Average results of a number of the writer's analyses appear also. All the analyses refer to the same kind of muscle as indicated. In the majority of cases

Comparison of Calculated and Actual Amounts of Protein in Muscle.

Species.	Total nitrogen in muscle.	Protein.		
		N × 6.25.	By difference.	Average of analyses according to the writer.
	per cent	*per cent*	*per cent*	*per cent*
Chicken	3.09*	19.3	19.0	16.6
Fish (halibut)	2.98*	18.6	18.4	16.5
Ox	3.46	21.6	21.5	16.6
Rabbit	3.39	20.8		16.3
Cat	3.38	21.1		17.8
Dog	3.25	20.2		17.4
Man	3.15	19.7		16.4

*Calculated from Atwater and Bryant's protein values. The remaining nitrogen analyses are by the writer.

the nitrogen was estimated in the same sample of muscle for which the protein was determined according to the writer's method. *The amount of protein calculated is seen to exceed that determined by actual analysis by about 15 to 20 per cent in nearly all cases. Continuance of the use of these usually employed methods of calculating muscle protein should therefore be discouraged.*

In order to put calculations of the caloric values of meat proteins on a more exact basis, the calories yielded by the pure muscle protein obtained in the analytical method should be determined. As it is possible to make preparations of meat extractives without

appreciable loss,[5] the caloric value of such preparations can also be obtained and correspondingly allowed for in metabolic work of this description.

The table also brings out the interesting fact that the actual protein content of muscle is usually quite constant for various species, even those as far separated zoologically as man and fish. In wasting diseases among human beings, however, values as low as twelve have been recorded. It seems possible that in such muscular disorders as myasthenia gravis the amount of protein muscle substance present might be found to bear a definite relation to the specific muscular seat of the disease.

[5] Janney, N. W., and Csonka, F. A., *J. Biol. Chem.*, 1915, xxii, 195.

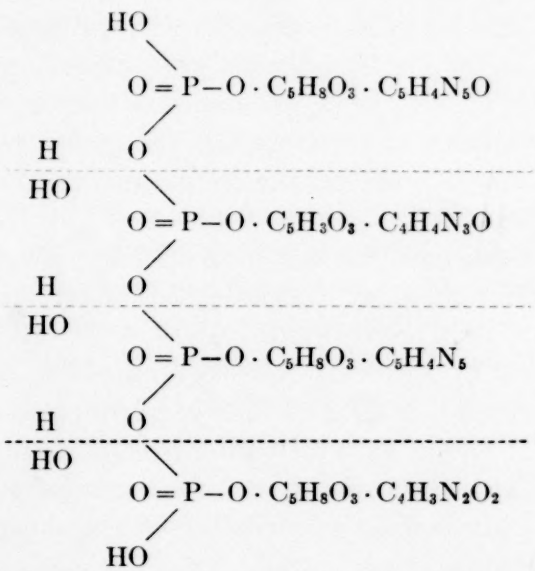
THE PARTITION OF PHOSPHORUS IN THYMUS NUCLEIC ACID.

By HILDEGARDE C. GERMANN.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University, Baltimore.)

(Received for publication, April 4, 1916.)

From a study of the optical changes which are brought about in yeast nucleic acid by the action of ferments, Levene and Medigreceanu[1] concluded that in the first phase of the enzymatic decomposition of the substance its four component nucleotides are produced:



Levene and Jacobs[2] showed also that yeast nucleic acid is initially decomposed in the same way by hydrolysis with dilute sulfuric acid, but that the two purine mono-nucleotides so formed are further decomposed, by prolonged acid hydrolysis, into phos-

[1] Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, 1911, ix, 389.

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1911, xliv, 1027.

phoric acid *d*-ribose and purine base, while the two pyrimidine mono-nucleotides remain unaltered.

Presumably any ribose nucleotide will decompose under the same conditions into its component mono-nucleotides, and by continued boiling with dilute acid the purine mono-nucleotides so formed will surely part with their phosphoric acid, but the pyrimidine mono-nucleotides will not. Therefore, a comparison of the easily split phosphoric acid with the total phosphoric acid obtainable from a compound nucleotide will give the ratio of its purine groups to its total nitrogenous groups, and the numerical index of the nucleotide may be ascertained.

Jones and Riley[3] have recently described a method by which this phosphoric acid ratio can be quickly and accurately determined. The results obtained may be interpreted from the following considerations.

A tetra-nucleotide (such as yeast nucleic acid) which contains two purine groups and two pyrimidine groups will give off half of its phosphoric acid by mild hydrolysis with sulfuric acid. So also will a di-nucleotide that contains one purine group and one pyrimidine group. But in such cases it is easy to show that the di-nucleotide contains but one purine group or even that it produces twice as much of the purine compound as could be obtained from a tetra-nucleotide.

A purine mono-nucleotide will give off all of its phosphoric acid while a pyrimidine mono-nucleotide will give off none of its phosphoric acid by mild acid hydrolysis. A tri-nucleotide would offer an excellent opportunity for the application of this method. If such a compound should contain one pyrimidine group and two purine groups it would give off two-thirds of its phosphoric acid; but if it should contain two pyrimidine groups and one purine group it would give off only one-third of its phosphoric acid by mild acid hydrolysis.

In case a nucleotide is contaminated with other substances (nucleosides, purine bases, or any compounds that do not produce phosphoric acid) its total phosphorus will be low but the numerical index of the nucleotide will not be affected by the presence of the impurity.

[3] Jones, W., and Riley, C., *J. Biol. Chem.*, 1916, xxiv, p. iii.

In case one is dealing with mechanical mixtures of different nucleotides, the numerical index found will not be a whole number. This was found true of a preparation that had been obtained by hydrolysis of yeast nucleic acid with ammonia at 115°. There was some reason for believing the substance to be a tri-nucleotide; but it is in fact a mixture of guanylic acid with a purine-pyrimidine di-nucleotide.[4] A dozen or more experiments with different preparations of the substance gave numerical indices from 2.35 to 2.68.

The application of this method to the animal nucleic acids can scarcely be without interest.

Thymus nucleic acid differs at two points from yeast nucleic acid as far as its fundamental groups are concerned. The carbohydrate group of thymus nucleic acid is a hexose group; that of yeast nucleic acid is a pentose group. The pyrimidine groups of thymus nucleic acid are thymine and cytosine groups; those of yeast nucleic acid are uracil and cytosine groups. According to Levene and Jacobs[5] both nucleic acids are tetra-nucleotides made up of two purine mono-nucleotides and two pyrimidine mono-nucleotides, but in the structure of the two nucleic acids their component mono-nucleotides are bound to one another at different points of juncture. It seems probable that the initial stage of acid hydrolysis would be the same in both cases; *viz.*, a splitting of the tetra-nucleotide into four mono-nucleotides; and one would suspect (after the analogy of yeast nucleic acid) that the two purine nucleotides of thymus nucleic acid would give up phosphoric acid by further acid hydrolysis while the pyrimidine nucleotides would not. One would therefore expect to find a tetra-nucleotide index. But this is not the case; the phosphorus values obtained lead sharply to a tri- or hexa-nucleotide index.

EXPERIMENTAL.

The sodium salt of thymus nucleic acid was prepared by the method of Neumann[6] and in order to remove a small amount of free phosphoric acid, the material was dissolved in hot water and treated with enough caustic soda to make a 1 per cent alkaline

[4] Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

[5] Levene and Jacobs, *J. Biol. Chem.*, 1912, xii, 411.

[6] Neumann, *Arch. f. Physiol., Suppl.*, 1899, 552.

solution. The warm solution was filtered from a little gelatinous phosphate (using a hot water funnel) and, after acidifying with acetic acid, the pale yellow fluid was warmed and poured into a large excess of alcohol. The precipitated sodium nucleate was washed by decantation with absolute alcohol and allowed to dry in a sulfuric acid desiccator.

Ten portions of the nucleic acid were weighed out. Two of these, intended for duplicate determinations of phosphoric acid were received into 500 cc. digestion flasks, and the other eight portions, which were to be used for determinations of partial phosphoric acid, were received into 100 cc. Erlenmeyer flasks which had been provided with corks and condensing tubes.

The determinations of total phosphoric acid were made as follows: The material was treated with a mixture of 5 cc. of concentrated sulfuric acid and 5 cc. of concentrated nitric acid, and boiled gently until all red fumes had been driven away and the liquid had become perfectly colorless. After cooling, 5 cc. of concentrated nitric acid were added and the product was again heated. As a third addition of nitric acid did not cause the production of brown fumes, the material was assumed to be sufficiently oxidized. The product was diluted to about 50 cc. with water, treated at the boiling point with 10 gm. of ammonium nitrate and an excess of 3 per cent ammonium molybdate, and the yellow ammonium phosphomolybdate was converted by the usual method into crystalline magnesium ammonium phosphate. The latter substance was allowed to dry for 48 hours at room temperature and was weighed.

The determinations of partial phosphoric acid were made as follows: The eight portions of nucleic acid were treated with 5 per cent sulfuric acid (20 cc. per gm. of nucleic acid) and after solution had been effected by heating, the condensing tubes were inserted and the flasks were submerged in a boiling water bath. Care was taken that the level of the liquid in the flasks was not below the level of the water in the water bath. The flasks were thus heated for ½ to 7 hours. As each flask was removed at the end of its time interval, the contents were made roughly alkaline with concentrated ammonia and, without waiting for the precipitation of guanine, the phosphoric acid was carefully precipitated with magnesia mixture. On standing over night the guanine and magnesium ammonium phosphate had settled sharply, leaving a perfectly clear, easily filterable fluid. This step of the procedure is absolutely necessary, because if an attempt be made to remove the guanine first (by precipitation with ammonia, as is easily done with the hydrolytic products of yeast nucleic acid), the guanine will be thrown out in so finely divided a form that it cannot be removed either by filtration or by centrifugation. But when the guanine and phosphoric acid are precipitated together, the fluid which contains the combined phosphoric acid can easily be filtered off.

The precipitate consisting of a mixture of guanine and magnesium ammonium phosphate was put into a 500 cc. Kjeldahl digestion flask and heated with a mixture of 5 cc. of concentrated nitric acid and 5 cc. of concentrated sulfuric acid until all organic matter had been destroyed. The determination of phosphoric acid in the oxidized mixture was then made by precipitation in turn as ammonium phosphomolybdate and magnesium ammonium phosphate, as described for the determination of total phosphoric acid. The results are given in Table I.

TABLE I.

Nucleic acid used.	Time.	Magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$.			
		Obtained.	Per gm. of nucleic acid.	Correction.	Remainder.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
0.7660	½	0.1015	0.132	0.006	0.126
0.7523	1	0.1303	0.173	0.011	0.162
0.6010	2	0.1326	0.221	0.022	0.199
0.7418	3	0.1774	0.239	0.033	0.206
0.7227	4	0.1856	0.257	0.044	0.213
0.7443	5	0.1977	0.265	0.055	0.210
0.5423	6	0.1506	0.277	0.066	0.211
0.9087	7	0.2549	0.280	0.077	0.203
0.4994	Total.	0.3027	0.606		
0.6245	"	0.3791	0.607		

In order that the significance of these results may be more apparent they have been constructed into a curve, one of whose coordinates is time, and the other, amount of magnesium am-

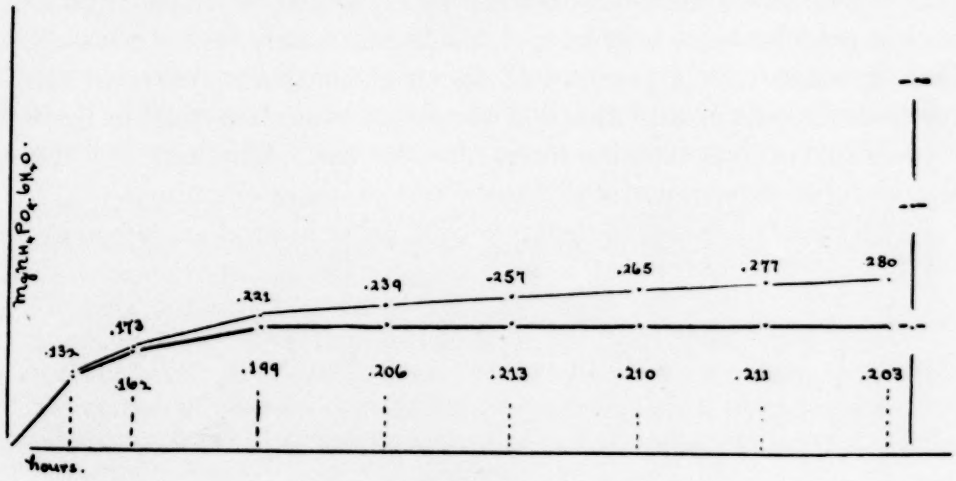


FIG. 1.

monium phosphate. The linear units have been arbitrarily chosen so as to bring out curvature as prominently as possible.

The principle of construction is that which Jones and Riley[3] have described in connection with yeast nucleic acid. The ordinates of points on the upper curve represent the quantities of magnesium ammonium phosphate actually obtained. This curve turns at the end of the 2 hour period and becomes a straight line slightly inclined to the axis of X, and its inclination shows that an amount of phosphoric acid corresponding to about 11 mg. of ammonium magnesium phosphate has been set free per hour. This amount was subtracted from each ordinate and the remainders were used as ordinates for the lower curve which ends in a straight line parallel to the axis of X and represents the amount of easily split phosphoric acid.

It will be noted that certain points do not fall exactly on the curve. This is due to uneven heating. It has been observed that when a flask dips too far below the level of the water in the water bath the experiment gives a point above the curve.

Nevertheless, the results as given are sufficiently accurate to show that of the phosphoric acid combined in thymus nucleic acid, one-third is easily split and two-thirds are firmly bound. This leads definitely to the conclusion that the number of phosphorus atoms in the molecule of thymus nucleic acid is divisible by three.

It is of interest to know whether this easily split phosphoric acid corresponds to purine groups, as is the case with yeast nucleic acid. But the amount of purine bases liberated from thymus nucleic acid by mild acid hydrolysis cannot easily be determined. The presence in the product of the undecomposed parts of the molecule interferes and there is danger of liberating purine bases in attempting to determine those already free. The matter offers considerable experimental difficulty but is being examined.

Experiments are also in progress with other animal nucleic acids.

THE DETERMINATION OF CREATINE IN MUSCLE. III.

By LOUIS BAUMANN and THORSTEN INGVALDSEN.[1]

(From the Chemical Research Laboratory, Department of Internal Medicine, State University of Iowa, Iowa City.)

(Received for publication, April 10, 1916.)

Muscle creatine is now determined colorimetrically after conversion into creatinine. As the Jaffé color test is not specific, it is questionable whether creatinine alone is responsible for this reaction. For this reason an apparent increase in the creatine content of muscle in feeding and perfusion experiments may in reality be due to some other substance. It therefore seemed desirable to develop a method for the isolation of the creatinine from muscle extract, preferably as the difficultly soluble potassium picrate salt. The creatinine content of the precipitate could then be estimated colorimetrically. Morris[2] has applied a somewhat similar procedure to the urine.

After considerable preliminary work the following technique was adopted. Approximately 5 gm. of hashed and well mixed muscle are weighed on the analytical balance and extracted according to Janney and Blatherwick.[3] After adding 5 cc. of 5 N hydrochloric acid and a pinch of granulated lead the water-clear extract is evaporated to dryness, at first over the flame and finally on the water bath.[4] The residue is taken up in hot water and filtered through a small filter. The washings and filtrate should not exceed 35 cc.

[1] The experimental data are taken from a dissertation submitted by Thorsten Ingvaldsen as a partial requirement for the degree of Master of Science, State University of Iowa, Iowa City.

[2] Morris, J. L., *J. Biol. Chem.*, 1915, xxi, 201.

[3] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

[4] Benedict, S. R., *J. Biol. Chem.*, 1914, xviii, 191. We have found, in corroboration of Benedict's statement, that this method is the most rapid for the conversion of urinary creatine into creatinine. It is accurate in the absence of sugar.

The solution is then neutralized with 10 per cent sodium hydroxide solution, using one drop of a 1 per cent alcoholic solution of methyl red as an indicator. The lead precipitate is filtered off and washed with hot water. The volume of fluid at this stage should not exceed 50 cc. 2 gm. of powdered picric acid and 0.5 gm. of potassium picrate are added to the solution and this is then heated on the water bath to 45°. After remaining at room temperature for 1 hour the sides of the beaker below the surface of the fluid are rubbed with a glass rod, and the solution is cooled in the refrigerator for 12 hours. The next morning the supernatant fluid is decanted through a Gooch crucible which has been washed with saturated picric acid solution.[5] The crucible is returned to the beaker containing the bulk of the precipitate. 15 cc. of 5 N sulfuric acid are poured into the crucible, about 60 cc. of water added, and the mixture is heated on the water bath for half an hour. The fluid in the beaker is then transferred to a 200 cc. volumetric flask. The crucible and contents of the beaker are well washed with hot water and the washings added to the flask. The contents of the flask are cooled with running water, made to volume, and filtered. 10 cc. of the filtrate and 0.9 cc. of the standard solution[6] are pipetted respectively into two 100 cc. volumetric flasks. 10 cc. of saturated picric acid solution and 3.5 cc. of 10 per cent sodium hydroxide solution are added to the unknown, and 15 cc. of picric acid and 2 cc. of the sodium hydroxide solution are added to the standard. After 10 minutes the flasks are diluted to the mark. The colorimetric reading is made with the standard set at 10 mm. The creatine content per 100 gm. of muscle can easily be computed with the help of the following formula.

$$\frac{1.16 \times 0.9 \times 10^3 \times 20}{\text{Reading of colorimeter} \times \text{Weight of muscle.}}$$

It is well to avoid a possible error due to uneven illumination of the colorimeter by reading the standard against itself. The correction, if any, must of course be inserted in the above formula.

[5] The filtrate should not give a positive Jaffé reaction upon the addition of 3 cc. of 10 per cent sodium hydroxide solution.

[6] The standard solution contains 1 mg. of creatinine in the form of creatinine zinc chloride per cc. of 0.1 N hydrochloric acid solution.

A comparison of the figures obtained by this procedure with those found by the direct methods of Janney and Blatherwick[3] or Baumann[7] shows close agreement.

On several occasions the picrate was decomposed with acid, the picric acid removed, and the nitrogen content of the solution determined. Here again the amount of nitrogen found agreed closely with the calculated quantity. This last step may become necessary when the presence of a substance other than creatinine which also forms an insoluble picrate is suspected.

EXPERIMENTAL.

Creatinine Can Be Quantitatively Precipitated with Picric Acid and Potassium Picrate.

To 20 cc. of a 0.1 N hydrochloric acid solution containing 20 mg. of creatinine, 2 cc. of 5 N sulfuric acid solution were added. The solution was neutralized and further treated as described above. This experiment was carried out in triplicate.

Found: 19.97, 19.97, and 19.97 mg. of creatinine.

Conversion of Creatine into Creatinine.

At the outset it was necessary to select a method of conversion which would avoid the use of an excess of acid.[8] Conversion by the autoclave method of Myers[9] was first tried. 2 cc. of 5 N sulfuric acid were added to the concentrated muscle extract (about 10 cc.) and this was then heated in the autoclave at 116–117° for 40 minutes. The figures obtained after precipitation are tabulated below. The direct controls were carried out according to the methods of Janney and Blatherwick or Baumann.

[7] Baumann, L., *J. Biol. Chem.*, 1914, xvii, 15.

[8] An excess of sodium picrate was found to be undesirable.

[9] Benedict, F. G., and Myers, V. C., *Am. J. Physiol.*, 1907, xviii, 397. Benedict, F. G., *J. Biol. Chem.*, 1914, xvii, 363.

Conversion by Myer's Method.

Dog muscle.		Beef muscle.	
Direct.	Precipitation.	Direct.	Precipitation.
mg.	*mg.*	*mg.*	*mg.*
373	352	485	474, 463, 464, 480, 487, 486, 467, 468, 475, 470, 478
		485	469
		412	386, 375, 373, 385, 390, 390, 383, 382, 384,* 385
		360	354, 343, 362, 367
		443	411, 418, 408, 417, 429, 414, 423, 436
		369	328, 318, 355, 356

* Determined directly after conversion without precipitation.

Acceptable results were obtained when the creatine was converted by boiling the concentrated extract (about 10 cc.) obtained from approximately 5 gm. of muscle, with 2 cc. of 5 N sulfuric acid for 3 hours under a reflux.

Beef muscle.	
Direct.*	Precipitation.
mg.	*mg.*
355	353, 357, 351, 355**
456	461, 455, 456**
464	458, 467, 459, 472, 461,** 474**

*Determined according to Janney and Blatherwick.
**Determined directly after conversion without precipitation.

This method was then compared with the autoclave method on a solution of pure creatine. 10 cc. of solution contained 19.76 mg. of pure anhydrous creatine.

Found: (a) 19.6 mg. 10 cc. boiled for 3 hours with 2 cc. of 5 N sulfuric acid.
(b) 19.14 " 10 cc. autoclaved with 1 cc. of 5 N sulfuric acid for 40 minutes at 117°.
(c) 18.92 " Same as (b).
(d) 19.46 " Same as (b) except that 2 cc. of acid were used.
(e) 19.62 " Same as (d).

Conversion, preliminary to precipitation, was finally carried out according to the method of Benedict.[4] It appeared to be the simplest method and as accurate as any of the others.

Conversion by the S. R. Benedict Method.

Direct.*	Direct.**	Direct.***	Precipitation.	Muscle.	
mg.	*mg.*	*mg.*	*mg.*		
449		445	433	Beef	XVI
426		404	405	"	XVII
		401	403	"	XVII
		402	401	"	XVII
450		428	422	"	XVIII
		419	433	"	XVIII
		421	427	"	XVIII
	509	532	490	"	XIX
		531	494	"	XIX
	476	479	479	"	XX
		479	483	"	XX
			483	"	XX
	417	431	431	Dog	XXI
		442	431	"	XXI
	476	515	522	Muskrat	XXII
			503	"	XXII
481	454	480	481	Beef	XXIII
			479	"	XXIII

*Baumann method.
**Janney and Blatherwick method.
***S. R. Benedict method without precipitation.

During the early period of this work it was occasionally necessary to purify the concentrated muscle extract with colloidal ferric hydroxide. The following experiment indicates that the iron precipitation did not remove creatinine from the solution. A converted muscle extract was diluted to a definite volume and the creatinine content of an aliquot portion determined. The remainder was accurately neutralized, precipitated with the colloidal iron solution, and the creatinine estimated in the filtrate.

Result: With iron 407, without iron 407 mg.

Analysis of the Precipitated Picrate.

The concentrated extracts from three 5 gm. muscle samples were converted, then united and precipitated with picric acid and

potassium picrate in the usual way. 4 gm. of picric acid and 0.8 gm. of potassium picrate were employed for this purpose. After 12 hours in the cold, the supernatant liquid was decanted through a Gooch crucible which had previously been washed with saturated picric solution. The bulk of the precipitate which had remained in the beaker was then washed with 30 cc. of ice-cooled saturated picric acid solution by decantation. The crucible was then connected with a clean flask and 10 cc. of 45 per cent sulfuric acid solution were poured on the precipitate in the beaker, and then filtered through the crucible. This process was repeated three times with fresh 10 cc. portions of acid. The first part of the filtrate was returned to the crucible to remove a small quantity of picric acid which had crystallized. The picric acid residue was finally washed with two 10 cc. portions of 5 N sulfuric acid solution. The remainder of picric acid, dissolved in the filtrate, was removed by thoroughly shaking with ether. This was repeated until the ether was colorless. The ether was removed with a pipette connected with a filter pump. The nitrogen in the picric acid-free solution was then determined according to Kjeldahl. It was necessary to boil 1 hour after the fumes began to be given off. The following results were obtained.

Beef muscle.	
Direct.	Calculated from the N found.
mg.	*mg.*
417	417
418	386, 403
482	481, 495
412	416

CONCLUSIONS.

1. Creatinine may be quantitatively precipitated from muscle extracts by means of picric acid and potassium picrate.

2. The values obtained by the precipitation method agree with those found by the direct methods of Janney and Blatherwick, S. R. Benedict, and Baumann.

IS AUTOLYSIS AN AUTOCATALYTIC PHENOMENON?

AN INTERPRETATION.

By H. C. BRADLEY.

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.*)

(Received for publication, March 27, 1916.)

In a recent paper bearing the above title Morse[1] shows that the curve of hydrogen ion concentration in an autolyzing tissue is of the logarithmic type and resembles in a general way the curve of proteolysis in the same tissues. This confirms the common observation that a tissue grows more distinctly acid to litmus as it autolyzes, and is in agreement with the observations of Kikkoji[2] and others regarding the formation of lactic acid in autolyzing tissues.

From these interesting data Morse draws the conclusion: "*Autolysis is an autocatalytic phenomenon, the products of digestion entering into the reaction as true catalyzers.*" To be autocatalytic the process must be, as Morse states earlier in the paper, one "*in which the process of enzyme action is accelerated by the products formed during the action of the enzyme and in consequence of it.*" But since *autolysis* is synonymous with *proteolysis* throughout the paper, and the enzyme in question is a proteolytic one, the conclusion would appear to affirm that the products of proteolysis—peptones, amino-acids, etc.—accelerate the tissue proteolysis. This conclusion we do not believe is intended by Morse.

In another place the statement is made that "*developing acidity*" induces "*greater and greater acceleration in the digestion rate.*" None of the curves which Morse cites to illustrate this, including curves of his own construction, show the slightest indication of acceleration in the *rate* of digestion. The curves rise most rapidly at the beginning and proceed at a diminishing rate of change until

[1] Morse, M., *J. Biol. Chem.*, 1916, xxiv, 163.

[2] Kikkoji, T., *Z. physiol. Chem.*, 1907, liii, 415.

equilibrium is attained. At this point—the maximum extent of digestion—the rate of change is zero.

It appears fair to conclude, therefore, that Morse does not mean exactly what he states in his conclusions in this paper, but that some confusion of ideas has occurred in his interpretation of his results. A part of this confusion appears to arise from a failure to differentiate sharply between the *rate of change* in the reaction and the *extent of change* or point of equilibrium. If the rate of change did actually increase, as Morse incorrectly asserts in the sentence quoted above, the reaction would indeed be an autocatalytic one. Since it does not, we believe the term is misleading and will only cause confusion if left unchallenged.

What we assume Morse intends to convey in this paper is this. *During the complex of reactions broadly termed autolysis, acids are known to appear. Their formation causes an increase in the acidity of the tissue as shown by the hydrogen ion concentration. Acidity renders the proteolytic enzymes active and is without effect upon the substratum. Proteolysis results. The products of acid formation thus activate the enzymes causing proteolysis, and in this sense a tissue considered as a whole, starts itself digesting its own proteins.*

Even assuming the above statement of the fact of activation to be correct, this is not autocatalysis *unless* the acids are produced by proteolytic enzymes, and of this no evidence is given. There is no evidence in the paper to substantiate the fact of enzyme activation which Morse assumes. Indeed the experiment is not calculated to determine this point, since the activity of an enzyme can only be judged by a digestion experiment. In spite of this lack of direct evidence, and because the curve of hydrogen ion concentration shows no sudden break during autolysis, Morse says "we should seek the simpler explanation, that the acidity developed acts catalytically, activating the enzyme in much the same manner that the hydrochloric acid of gastric digestion activates; for there is nothing to indicate a sudden alteration in the $H^{\cdot}$ content in the curves." The lack of sudden alteration in the $H^{\cdot}$ content of autolyzing tissue is merely proof that the proteins are still unsaturated with acid and are serving like buffer salts into which considerable acid may be poured before a sudden alteration of the $H^{\cdot}$ will occur. Strong HCl may be added to a 20 per cent liver

digest to a titratable strength of 0.04 N without increasing the $H^{\cdot}$ content above that expressed by about P_H 6.00. This is far more acid than is formed in the normal control digest and produces a far more rapid and complete proteolysis.

There is no essential difference between development of acidity by the formation of lactic and fatty acids in a digest and the introduction of such acids artificially from without. The same mechanism, determining the rate and extent of digestion, must be operative in both cases. Morse believes this mechanism to be activation of the proteolytic enzymes alone, at least in the normal tissue. On the other hand, we have presented data[3] which throw some doubt on activation as of major importance in this reaction. It is entirely possible that an enyzme such as the β protease found by Hedin[4] in the spleen, active only in acid media, is also present in the liver. Whatever the nature of the enzyme we know that it is active in a $H^{\cdot}$ concentration between P_H 7.3 and 6.7, since most livers show proteolysis within these concentrations. The enzyme is thus active at a fairly low $H^{\cdot}$ level. The fact that casein and peptone digest no faster in an acidified digest than in a normal control indicates that further additions of acid to the already active enzyme have little or no effect upon its activity. Yet such additions of acid have a tremendous effect in increasing the rate and extent of digestion of the liver proteins. This is, we feel, rather direct proof of alteration of the mass of the substratum.

The data which Morse has presented and which we assume to be correct may therefore be interpreted in quite a different manner without doing violence to the facts developed by previous work in the field and without involving confusion.

1. At a P_H of about 7.00 ± the liver protease is active as shown by the fact that some proteolysis goes on at that level.

2. Acid develops in the autolyzing mixture, rapidly at first, reaching its maximum concentration in about 3 days.

3. Accompanying this increased acidity and caused by it, is a corresponding change in the protein substratum of the tissue. If much acid is produced the extent of digestion will be large; if little is produced digestion will reach equilibrium at a lower level.

[3] Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113.

[4] Hedin, S. G., *J. Physiol.*, 1904, xxx, 155.

4. The mass of substratum will determine the point of equilibrium, but the amount of acid will determine the mass of substratum.

5. There is nothing in the data and curves of autolysis thus far presented in the literature to suggest an autocatalytic reaction.

A NEW SALT OF URIC ACID AND ITS APPLICATION TO THE ANALYSIS OF URIC ACID AND PHENOL.

PRELIMINARY PAPER.

By J. LUCIEN MORRIS.

(*From the Laboratory of Biological Chemistry, Washington University, St. Louis.*)

(Received for publication, April 17, 1916.)

Some time ago the writer had occasion to use the colorimetric analysis of uric acid as a routine procedure in a large number of determinations. The small amount of material available, and the large amount of polyphenols or other interfering substances[1] present emphasized certain difficulties of the method. From time to time since then the failure of an analysis without any apparent cause has compelled interest in those factors which may interfere with a perfect result. Probably the most constant element of difficulty in this method is, as in all work with uric acid, very closely associated with its marked insolubility. But the effect of the presence of various salts, even in very small amounts, upon the colorimetric determination, is noticeable. The observations so far made do not justify a general conclusion as to the nature of the interference with the color development, but there is considerable evidence that it is due to the formation of urates and the uric acid is so removed from the reaction with the phosphotungstic reagent. Whether this is true in all cases is still a question, but the writer believes that he has shown it definitely for the marked interference due to the presence of zinc salts in the uric acid solution during preparation for the color reaction, when the amount of color may be very seriously diminished or entirely absent, or after the development of the color, when fading is much accelerated.

Out of the recognition of such an action of zinc upon uric acid grew the idea that possibly the compound so formed might be

[1] The work referred to was on 24 hour rat urines.

useful in precipitating uric acid. Upon searching the literature no mention of a zinc urate was found. Attempts were made at precipitating it, but with little encouragement. Various zinc salts were added to uric acid and urates, and though precipitates were formed when the solution was made alkaline, there was no evidence that the precipitates consisted of any substance other than zinc hydroxide, carbonate, or phosphate, according to the medium in which the uric acid had been dissolved. Nor did analysis of the filtrates from such precipitates give any indication of the amount carried out of solution, because of the great uncertainty of the value of any results obtained in the presence of such rapid fading as is caused by zinc compounds.

At this point Ganassini's reaction[2] came to my attention. The directions are to add soluble zinc salts to a weakly alkaline solution of uric acid, thus obtaining a white precipitate which turns blue on the filter paper, due to oxidation in the presence of air. By the use of this qualitative test, the writer was able to detect the presence of small amounts of uric acid in considerable volumes of water. This strengthened the belief that uric acid was precipitated by zinc, though it was possible that it was carried down mechanically by the flocculent precipitates of zinc carbonate, zinc hydroxide, or zinc phosphate. In either case, the zinc must evidently be removed from the filtrate if the colorimetric method,[3] or, for that matter, any of the other methods, be used to determine how much uric acid had been removed from solution. This was accomplished by precipitating it as sulfide after acidifying with acetic acid. (This precipitation which is generally so troublesome, was made entirely satisfactory by adding about 0.25 to 0.5 gm. of bismuth carbonate to the hot solution. Then upon saturating with hydrogen sulfide, the finely divided white zinc sulfide is apparently enveloped by the bismuth sulfide, and when enough bismuth is present, the precipitate of mixed sulfides settles from a clear solution. The subsequent filtration is then as easy as that of bismuth sulfide

[2] Ganassini, D., *Bol. Soc. med. chir.*, Pavia, 1908, i; reviewed, *Biochem. Zentr.*, 1908–09, viii, 250.

[3] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619. The phosphate-uric acid standard and the KCN substitution for H_2S as well were used as modifications of the Folin-Denis method throughout this work.

alone.) The filtrate was evaporated over a flame, an air current being passed over the surface of the liquid to hasten the evaporation as well as the escape of the hydrogen sulfide. When the volume had been diminished to 10 to 15 cc., the uric acid was determined in the usual colorimetric manner. The results showed conclusively that the uric acid was largely removed from its solution in sodium carbonate, lithium carbonate, or the phosphate mixture of Benedict and Hitchcock[3] by the zinc precipitation. Precipitation from a pyridine solution was also very evident, though the presence of pyridine made the removal of the zinc, and therefore the subsequent determination, less satisfactory.

Attempts were made to obtain the compound of zinc and uric acid for identification. Such efforts have so far proven uniformly unsuccessful. The reason is that the nature of the compound is so characteristically that of other zinc salts, *e.g.*, the phosphate, the carbonate, and the hydroxide, that it cannot be obtained separate from these compounds. The reaction must be neutral, or, better, slightly alkaline to precipitate the zinc-uric acid compound, and this is the condition favoring the precipitation of the zinc in the form of its compound with the alkali used. The same state of affairs exists of course when pyridine or other organic base is the alkali. If the precipitate is then treated with acid or alkali, the uric acid is set free from the compound either in the form of the acid or its alkali salt. This close association with any or all zinc compounds that may be present has stood in the way of every attempt at its separation or purification. This fact, annoying as it has proven, is in itself strong evidence that the compound is zinc urate.[4]

While trying to separate zinc urate in a pure state, the conditions for its most complete precipitation were obtained. They are as follows: Acidify the solution containing uric acid with acetic acid and add 10 per cent zinc acetate solution in a quantity in excess of the amount required to precipitate the phosphates present. Stir well and then add saturated sodium carbonate solution until the reaction is alkaline to litmus. There will be a flocculent precipitate of zinc carbonate and zinc urate (also phosphate if present). If it is desired to determine the amount of uric acid which

[4] Further attempts are being made to obtain the salt in the pure state.

is contained in this precipitate, filter or centrifuge, and dissolve the precipitate in a small amount of dilute acetic acid; heat to boiling, add a small amount of bismuth carbonate, and saturate with hydrogen sulfide. Filter, drive off the hydrogen sulfide by heating, concentrate to 10 to 15 cc., and determine the uric acid content colorimetrically.

That this procedure will remove a very small amount of uric acid from a considerable volume of liquid is shown by the following examples. 5 cc. of the phosphate-uric acid standard, containing 1 mg. of uric acid and five drops of concentrated acetic acid, were added to 1 liter of water; then 3 cc. of 10 per cent zinc acetate, and saturated sodium carbonate, added drop by drop, until alkaline to litmus. Filtration, solution of the precipitate in acetic acid, precipitation of zinc sulfide, and evaporation as above described followed. A colorimetric determination in the final step showed $\frac{20.0}{21.5} = 0.93$ mg. recovered. In a similar experiment with diluted urine, 3 cc. made up to 1,000 cc., gave a colorimetric value of $\frac{20.0}{19.3} = 1.04$ mg., while a determination directly upon 3 cc. of the same urine gave a value of 1.16 mg. This means that the solubility of zinc urate is of the order 0.07 mg. in 1,000 cc. of water containing a small amount of sodium carbonate and zinc carbonate, and 0.12 mg. in 1,000 cc. of such a mixture plus 3 cc. of urine.

A precipitation so nearly or quite complete seemed worth while in its application both to the identification of uric acid in very dilute solutions and in the removal of uric acid from solutions to make way for the determination of other substances reacting with the phosphotungstic reagent. As a means of removing the uric acid from urine preparatory to phenol determinations, it has proven fully as efficient as the acid silver lactate and colloidal iron precipitation followed by removal of the excess silver, used by Folin and Denis,[5] and certainly more rapid. The conditions maintained by the writer for this precipitation in undiluted urine are as follows: To 2 to 4 cc. of urine add 1 cc. of 10 per cent zinc acetate solution and enough acetic acid to dissolve the zinc phos-

[5] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.

phate formed. Then add saturated sodium carbonate solution, drop by drop, until alkaline, and filter. Wash with about 10 cc. of water to which have been added a few drops of sodium carbonate. To remove zinc from the filtrate add about 0.5 gm. of calcium carbonate, bring to a boil, and after adding 1 cc. of 10 per cent sodium oxalate, continue boiling about half a minute. Filter and wash with about 10 cc. of water. Cool and acidify with oxalic acid. Add the phenol reagent and sodium carbonate and make up to volume with lukewarm water in the usual way. Since the final volume of the phenol solution depends upon the amount of wash liquids added, it is necessary to add an approximately equal volume of water to the phenol standard before developing the color in it. Proceeding as described with standard solutions of uric acid and phenol, the results shown in the table were obtained.

TABLE I.

Separation of Uric Acid and Phenol by Precipitation of Zinc Urate.

No. of analyses.	Uric acid present.	Phenol present.	Phenol present after precipitation.
	mg.	*mg.*	*mg.*
3	1.00	0.00	None or negligible trace.
4	0.00	1.00	0.98 0.99 0.99 0.97
3	1.00	1.00	0.99 0.98 1.01

With 5 cc. of the standard phosphate-uric acid solution after treatment there was no more color than in a blank of phenol reagent and carbonate; 10 cc. of the phenol standard (1 mg. of phenol) subjected to the same read 97 to 99 per cent of the true value; and solutions containing 1 mg. of uric acid and 1 mg. of phenol gave color reactions equivalent to 98 to 101 per cent of 1 mg. of phenol.

Since the separation of the zinc urate is very complete and practically instantaneous even from solutions of low concentration of uric acid, and room temperature serves equally as well as higher

temperatures, it has seemed probable that the precipitation may prove useful in determining uric acid in urine and blood. This is now being investigated by the writer, who hopes soon to publish conditions bearing upon this practical application of the new zinc urate precipitation.

CHEMICAL AND PHYSICAL ANALYSIS OF BLOOD IN THIRTY NORMAL CASES.

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(Received for publication, April 6, 1916.)

INTRODUCTION.

Although normal values for various blood constituents and blood serum constants have been published within the last few years by various workers, there is no record where they have all been determined in the same individual and on blood taken at one time. Furthermore, the values given in the literature disagree more or less; this may be due to the different methods in use by various workers, and also to the nature and time of the preceding meal or meals. It was therefore decided to adhere to some fixed standard conditions which could easily be maintained in similar work on patients in the hospital.

Standard Conditions.—(1) Breakfast, consisting of one egg, one bread roll with butter, and one cup of coffee with 20 cc. of milk (the regular hospital breakfast). (2) Blood is taken 3 hours thereafter. (3) Determinations are started at once. (4) The same standard methods are always used.

Determinations Made.

Blood, Chemical.—Solids, coagulable nitrogen, non-coagulable nitrogen, ammonia nitrogen, urea nitrogen, residual nitrogen, uric acid, creatine, creatinine, chlorides, glucose, fat, cholesterol.

Blood, Physical.—Conductivity, hydrogen ion.

Blood, Biological.—Red count, white count, corpuscular volume, hemoglobin, Wassermann test.

Plasma.—Alkaline reserve, chlorides, nitrogen.

Serum.—Specific gravity, freezing point, conductivity, refraction, nitrogen.

Cases Studied.

Normal men and women in robust health and in active pursuits (teachers, medical students, laboratory workers, business men, laborers, and others), ranging in ages from 17 to 60 years, volunteered the material on which this study is based. Cases 22 to 26 were hospital patients (simple fracture cases from the surgical service of Professor John A. Hartwell, to whom we are indebted for allowing us to study these cases), examined on the morning of their discharge. Physical examinations and urine analyses were made on these and on all other cases of the series.

Time of Taking Blood.—In a preliminary investigation in which normal blood was studied after a 12 hour fast and again 2 or 3 hours after a meal (comprising one egg, one bread roll with butter, coffee, and milk) the values were always found to lie within the normal range. In this series, for convenience, blood was removed 3 hours after a breakfast as above.

Method of Blood Letting.—A few drops of 2 per cent novocaine were injected for local anesthesia. A short but large caliber needle was introduced into the median vein, and 75 cc. of blood were allowed to flow out in 3 to 5 minutes. This was collected in sterile, clean, Erlenmeyer flasks, 50 cc. being taken in one containing 0.5 gm. of potassium oxalate to prevent clotting, and 25 cc. in another flask without oxalate. The latter was allowed to stand in the ice box 2 to 3 hours after clotting, preliminary to pouring off serum.

Blood Counts.—Red and white counts were made on all cases at the time of phlebotomy. Total counts only are tabulated, differentials having been made in those cases exceeding 9,000 white corpuscles per c.mm., to exclude possible abnormal conditions.

Hemoglobin.—This was estimated by Dare's hemoglobinometer,[1] which appears to be quite accurate for the higher readings presented by normals.

Corpuscular Volume.—In conjunction with the preceding determinations that of corpuscular volume seems valuable. This observation is made by immediately centrifuging the freshly drawn and well mixed oxalated blood in a calibrated capillary

[1] Dare, A., *Phila. Med. J.*, 1898, vi, 557.

tube. These were made[2] 10 cm. long to be conveniently carried in the jacket of the regular 15 cc. conical centrifuge tubes. A large elastic band is slipped about the ends, and it is then centrifuged for 5 minutes at 2,500 R. P. M. The red cells are packed to one end, and resting upon them the buffy layer of the less dense leukocytes may be seen. Readings of the total cellular volume of the blood are made directly in per cent.

Part I. Chemical Analysis.

After trial of various methods proposed in the literature the following were found most suitable and were used throughout this investigation.

Blood Solids.—One drop of blood was caught on a small piece (2 sq. cm.) of dried blotting paper, which was at once placed in a wide-mouthed weighing bottle (the stopper being inserted at once to avoid evaporation) and weighed. The stopper was then tilted, the whole placed in an air bath at 80–90°C., and dried to constant weight.

The solids range from 21 to 24 per cent, two cases only being a trifle outside of these limits (see table). From this series the value of 20 per cent as given by Myers and Fine[3] seems too low.

Total Nitrogen of Blood, Plasma, and Serum.—1 cc. of each (uniformly delivered and washed from a standardized 1 cc. bulb pipette) was used in the regular Kjeldahl process. The titrations were done with 0.05 N solutions.

Total Nitrogen.

	Gm. per 100 cc.
Blood*	3.0–3.7
Plasma	1.1–1.4
Serum	1.2–1.4

*The value of 3.0 gm. of nitrogen in 100 cc. of blood as given by Myers and Fine,[3] seems to be the low level for normals.

Non-Protein Nitrogen.—In place of using either methyl alcohol or trichloroacetic acid for the precipitation of the protein substances, we use an acid mercuric chloride solution. Although

[2] Made by Emil Greiner Co., New York.

[3] Myers, V. C., and Fine, M. S., *The Post-Graduate*, New York, 1914–1915.

bichloride of mercury has been used as a protein precipitant ever since Schenck[4] suggested it, it has never been used in connection with non-protein nitrogen determinations in blood. The reason for this is that Schenck's 0.8 per cent hydrochloric acid and 2 per cent bichloride solution gives almost invariably a very cloudy filtrate, containing nitrogen other than non-protein. On trying stronger solutions of both the acid and the mercuric chloride, it was found that 5 per cent hydrochloric acid and a 5 per cent solution of bichloride always gives a water-clear filtrate. The advantage of this precipitant is that a perfect separation of protein from non-protein substances is obtained with only one filtration, whereas in the method of Folin and Denis[5] with methyl alcohol and zinc chloride, as well as in Greenwald's[6] with trichloroacetic and kaolin, two filtrations must be made, one after treatment with each reagent.

Furthermore, the time required for complete precipitation is reduced to a few minutes.

Method.—5 cc. of blood are pipetted into a small Erlenmeyer flask. 5 cc. of water are added to lake the blood, then 10 cc. of 5 per cent HCl solution; the mixture is well shaken, and 10 cc. of 5 per cent mercuric chloride solution are added, making in all exactly 30 cc. This is mixed well, then centrifuged thoroughly. The clear supernatant liquid is filtered through a small, dry filter paper; of the filtrate 20 cc. are taken for a micro-Kjeldahl, using 5 cc. of concentrated sulfuric acid and about 1 gm. of potassium sulfate. (Boiling for $\frac{1}{2}$ hour is sufficient because the excess mercury from the precipitating agent serves here as a very efficient catalyst.)

The ammonia is determined by aeration and Nesslerizing, or, as done in this series, by distilling over into 20 cc. of 0.025 N sulfuric acid, and titrating back with 0.025 N sodium hydroxide, using Congo red as indicator.

The values range from 30 to 45 mg. per 100 cc. of blood. Three cases only went a trifle higher (see Composite Table). All values above 50 mg. should be regarded as pathological.

The following is for comparison of values obtained by various workers.

[4] Schenck, F., *Arch. ges. Physiol.*, 1894, lv, 203.

[5] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

[6] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

Non-Protein Nitrogen.

	Mg. per 100 cc. of blood.
This series	30–45
Folin and Denis[7]	22–37
Greenwald[6]	30
Bang[8]	19–39
Taylor and Hulton[9]	25–28
McLean and Selling[10]	23–44
Myers and Fine[3]	25–30
Hohlweg[11]	40–61

Urea Nitrogen.—Marshall's[12] urease method was used for decomposing the urea into ammonium carbonate; Folin's[5] aeration and colorimetric method for determining the same. The Duboscq colorimeter was used in all cases.

Values obtained range from 15 to 25 mg. of urea nitrogen in 100 cc. of blood. Only two cases fell below 15 mg. and none exceeded 25 mg. (see Composite Table).

Urea Nitrogen.

	Mg. per 100 cc. of blood.
This series	15 –25
Folin and Denis[7]	12 –27
Schwartz and McGill[13]	10.8–25.2
Bang[8]	6 –20
Myers and Fine[3]	12 –15
McLean and Selling[10]	12 –27

Ammonia Nitrogen.—Using 5 cc. of blood, the ammonia was aerated and determined colorimetrically according to the method of Folin and Denis.[5] The values obtained range from 0.4 to 0.75 mg. in 100 cc. of blood (see Composite Table).

Uric Acid.—The colorimetric method of Folin and Denis[14] was used, with the modification suggested by Benedict.[15]

[7] Folin and Denis, *J. Biol. Chem.*, 1913, xiv, 29; 1914, xvii, 487.
[8] Bang, I., *Biochem. Z.*, 1915, lxxii, 104.
[9] Taylor, A. E., and Hulton, F., *J. Biol. Chem.*, 1915, xxii, 63.
[10] McLean, F. C., and Selling, L., *J. Biol. Chem.*, 1914, xix, 31.
[11] Hohlweg, H., *Med. klin. Woch.*, 1915, xi, 331.
[12] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 487.
[13] Schwartz, H., and McGill, C., *Arch. Int. Med.*, 1916, xvii, 42.
[14] Folin and Denis, *J. Biol. Chem.*, 1912–13, xiii, 469.
[15] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

The results seem to show that normal values of uric acid range between 1.0 and 3.5 mg. in 100 cc. of blood. Two cases fell below 1 mg. (see Composite Table).

The following is for comparison of values obtained by various workers.

Uric Acid.

	Mg. per 100 cc. of blood.
This series	1.0–3.5
Folin and Denis[7]	0.7–3.7
Maase and Zondek[16]	2.5
Myers and Fine[3]	1.0–2.0

Creatine and Creatinine.—Folin's[17] method was used for determining both the creatine and the creatinine.

Most of the values obtained for preformed creatinine are 0.1 mg. or less in 100 cc. of blood. It was not deemed worth while to estimate below 0.1 mg., which value is therefore given in the table whenever the actual amount was found to be this or less. Four cases were a trifle higher.

For creatine the values range between 3 and 6.5 mg. in 100 cc. of blood (see Composite Table).

Creatine and Creatinine.

	Mg. per 100 cc. of blood.	
	Creatine.	Creatinine.
This series*	3.0– 6.5	0.1–0.5
Folin and Denis[7]	5.2– 8.1	1.1–1.4
Myers and Fine[3]	5.0–10.0	1.0–2.0

*From this investigation it appears that creatinine is present normally in traces only and any value exceeding 1 mg. should be considered pathological.

Residual Nitrogen.—This value was obtained by subtracting from the total non-protein nitrogen the sum of the nitrogen values for urea, ammonia, uric acid, creatine, and creatinine. This undetermined nitrogen includes the amino-acid nitrogen. The values obtained range from 10 to 25 mg. in 100 cc. of blood. Only three cases fell below this value (see Composite Table).

[16] Maase, C., and Zondek, H., *Münch. med. Woch.*, 1915, lxii, 1110.
[17] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

Bang[8] reports 3 to 22 mg. "amino-acid nitrogen" per 100 cc. of blood, but his figures indicate only the difference between total non-protein and urea nitrogen.

Van Slyke's[18] method for the quantitative determination of the amino-acids present was not used in this series, because it would necessitate the further withdrawal of more blood than we thought advisable.

Blood Sugar.—The original method of Lewis and Benedict[19] was chosen because of its accuracy and simplicity.

The values obtained range between 50 and 120 mg. per 100 cc. of blood (see Composite Table).

The following is for comparison of results obtained by various workers.

Sugar.

	Mg. per 100 cc. of blood.
This series	50–120
Lewis and Benedict[19]	90–110
Michaelis[20]	90–130
Strouse[21]	40–120
Taylor and Hulton[9]	50–150
Naunyn[22]	70–100
Liefmann and Stern[23]	70–110
Hollinger[24]	70–100
Bang[25]	100–110
Frank[26]	80–110
Rolly and Oppermann[27]	62– 88
Kowarsky[28]	50–110
Freund and Marchand[29]	55–120

[18] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.
[19] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.
[20] Michaelis, L., *Biochem. Z.*, 1914, lix, 166.
[21] Strouse, S., *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 211.
[22] Naunyn, B., Diabetes Mellitus, Vienna, 2nd edition, 1906.
[23] Liefmann, E., and Stern, R., *Biochem. Z.*, 1906, i 299.
[24] Hollinger, A., *Deutsch. Arch. klin. Med.*, 1908, xcii, 217.
[25] Bang, *Biochem. Z.*, 1913, lvii, 300.
[26] Frank, E., *Z. physiol. Chem.*, 1910–11, lxx, 129.
[27] Rolly, F., and Oppermann, F., *Biochem. Z.*, 1912–13, xlviii, 187.
[28] Kowarsky, A., *Deutsch. med. Woch.*, 1913, xxxix, 1635.
[29] Freund, H., and Marchand, F., *Deutsch. Arch. klin. Med.*, 1913, cx, 120.

Fat and Cholesterol.—The methods of Kumagawa and Suto,[30] Rosenthal and Trowbridge,[31] and Bloor[32] are very time-consuming. We have therefore modified the Soxhlet method for fat in milk, so that it may be applied to blood. The ordinary method necessitates drying the material on a paper coil at 90–95°C. This cannot be done with blood, because of the ease with which the unsaturated fatty substances in blood are oxidized in air. In order to avoid oxidation, we consequently dry the blood in vacuum over P_2O_5. Furthermore, great stress must be laid on the use of anhydrous ether for the Soxhlet, otherwise foreign substances will be extracted along with the fats and cholesterol.

Method.—5 cc. of blood are allowed to run slowly from a pipette onto a very loose coil of fat-free absorbent paper; this is dried over night in a vacuum desiccator over P_2O_5 at 50–60°C. The coil is then extracted with anhydrous ether in a Soxhlet for 5 hours. The ether is evaporated from the extracted material, leaving a residue not in the least contaminated by pigments. It is dried to constant weight *in vacuo* over P_2O_5. The residue thus obtained consists of fatty substances plus cholesterol. To determine the cholesterol, it is taken up with 5 cc. of chloroform, to which are added 2 cc. of acetic anhydride and 0.1 cc. of H_2SO_4, as directed by Autenrieth and Funk,[33] and also by Bloor.[34] The solution is made up to 10 cc. with chloroform, allowed to stand in the dark for 15 minutes, and then compared in a Duboscq colorimeter with a similarly treated standard solution of cholesterol in chloroform (5 mg. of cholesterol in 10 cc. are a good strength for comparison).

Values obtained for cholesterol range from 30 to 60 mg. in 100 cc. of blood (see Composite Table).

Subtracting the value of the cholesterol from that of the total ether extract gives the amount of fat plus fatty acids present (also lipoids present in traces). Values for fat plus fatty acids range from 60 to 160 mg. per 100 cc. of blood. One case was as high as 320 mg. (see Composite Table).

This method has the following advantages over those pre-

[30] Kumagawa, M., and Suto, K., *Biochem. Z.*, 1908, viii, 212.
[31] Rosenthal, H., and Trowbridge, P. F., *J. Biol. Chem.*, 1915, xx, 711.
[32] Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.
[33] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.
[34] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

viously in use: (1) Both fats and cholesterol may be determined in the same 5 cc. portion of blood. (2) It does away with the repeated ether extraction and washing in separatory funnels. (3) The actual time of manipulation is only 30 minutes, the drying and extraction needing no attention.

Chlorides.—Determinations were made both upon blood and plasma, using the method of McLean and Van Slyke.[35]

450 to 500 mg. of chlorides in terms of NaCl were obtained in 100 cc. of blood, and 560 to 640 mg. in 100 cc. of plasma (only four cases fell a little below these values; see Composite Table).

Previously reported results by other workers agree well with these averages.

Total Chlorides as NaCl.

	Mg. per 100 cc. Blood.	Plasma.
This series	450–500	560–640
McLean and Van Slyke[35]	490	597–614
Rogée and Fritsch[36]	450	
Myers and Fine[3]		650

Alkaline Reserve of Blood.—Use was made of Van Slyke's[37] method for determining the carbon dioxide capacity of plasma at alveolar tension. From the burette readings the volume percentage of carbon dioxide bound as carbonate by the plasma was obtained by reference to Van Slyke's table. In his opinion, normals show 53 to 77 volume per cent of CO_2 chemically bound by plasma, figures lower than 50 per cent in adults indicating acidosis. All values obtained in this series agree with the above. The majority lie between 58 and 68 volume per cent, and none is below 55 (see Composite Table).

Part II. Physical Analysis.

Conductivity of the Circulating Blood.—A cell was devised[2] for determining the conductivity of the blood as it exists in circulation. It consists of three pieces: a center piece B, which is a tube $1\frac{1}{2}$ inches long and $\frac{1}{2}$ inch outside diameter, and having a capacity

[35] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.
[36] Rogée, H., and Fritsch, C., *Biochem. Z.*, 1913, liv, 53.
[37] Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 7.

of 4 cc.; into this are fused two glass tubes D which carry the platinum poles E at their terminals and offer an electrical capacity between 1 and 2. Into these tubes is put mercury which serves to connect the poles with the wires F leading from the Kohlrausch bridge. A and C are ground glass hollow stoppers having at their apices small openings, and are ground to fit the Luer needle used in taking the blood.

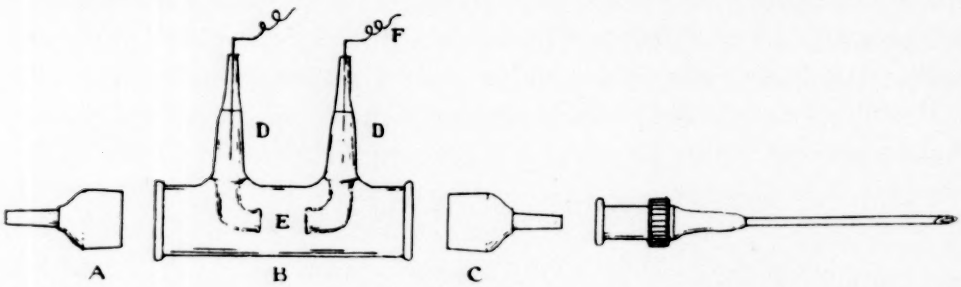

FIG. 1.

Method of Procedure.—After the needle has been inserted into the vein, the cell is immediately fitted to the other end and the blood flows freely through the apparatus, no air bubbles remaining about the vertical poles. As the blood leaves the cell it is led into flasks to be further used in the chemical analysis. While the blood is thus passing through the cell, readings are made continually using the Kohlrausch bridge.[38] It is found that the readings which are low at first rise quickly and attain a constant value in about 1 minute, as soon as the cell has been warmed to the temperature of the blood.

The values obtained are about 60 per cent less than serum conductivity values, due probably to the additional resistance offered by the blood corpuscles and fibrinogen. The conductivities for whole blood in circulation range from $\Lambda = 37–64$, 80 per cent being in the range $\Lambda = 42–54$ (see Composite Table).

Serum Conductivity.—Special U-shaped tubes were devised[2] so that determinations could be performed on as little as 0.5 cc. of serum. All readings were made at 25°C. with a Kohlrausch bridge. The results show the conductivity $\Lambda = 112–125$, 90 per cent being between $\Lambda = 115–122$.

[38] Made by Leeds Northrup Co., Phila.

Hydrogen Ion.—The gas chain method of Nernst as modified by Michaelis[39] (V-shaped gas electrode with stationary hydrogen) was used. The blood is allowed to run from the needle directly into the right arm of the electrode, having in the left arm, in the order named, a ground glass stopper holding the platinum pole, the hydrogen gas, the 0.85 per cent NaCl, and a few particles of hirudin. When the right arm is filled with blood, it is stoppered with a ground glass stopper so that all air is removed. The whole is well mixed by inverting it about thirty times. The stopper of the right arm is now removed and the cell is connected with the standard calomel electrode (by means of a wick saturated with KCl solution), the Leeds and Northrup potentiometer, a sensitive reflecting galvanometer, etc., and the values are read every 2 minutes. In every case equilibrium is established within 10 minutes.

The values for P_H in this series range from 7.52 to 7.60 at 22°C. (see Composite Table). Michaelis[39] gives a value of 7.56 at 18°C., Hasselbalch,[40] 7.52 at 18°C., and de Corral,[41] 7.59 at 14°C.

Specific Gravity.—The density of serum at 25°C. as compared with water was determined by weighing each in a standardized pyknometer, having a capacity of a little less than 1 cc.

The specific gravity values range from 1.0260 to 1.0300, the majority being between 1.0270 and 1.0290.

Cryoscopy.—(A relative measure of crystalloids present.) The freezing point determinations were made with the Beckmann apparatus, with the usual precautions as to temperature of cooling bath, stirring, supercooling, inoculation, tapping, etc. Each determination was preceded and followed by a zero determination (using freshly boiled distilled water). The values range between −0.557 and −0.577°C. (see Composite Table).

Refractive Index.—(A relative measure of proteins present.) The Zeiss immersion refractometer was used for this determination. From the readings obtained at 25°C. was subtracted the refraction due to water, and the resulting figure was multiplied by 1,000, in order to record the values in customary units (Δ Nd $\times 10^3$).

[39] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.
[40] Hasselbalch, K. A., *Biochem. Z.*, 1912, xxxviii, 77.
[41] de Corral, M. J., *Biochem. Z.*, 1915, lxxii, 1.

With very few exceptions the normal sera show values of 16.2 to 18.5 (see Composite Table). Butterfield[42] gives values of 16.8 to 18.0 obtained on five normal individuals.

SUMMARY.

1. The blood of thirty normal individuals (males and females of ages ranging from 17 to 60) was analyzed for the various chemical constituents and physical constants, using the modern micro-methods, thus giving a fairly complete picture of blood in health.

2. Twenty-eight determinations were made on each sample, requiring in all only 70 cc. of blood.

3. Among the values found, the following should be mentioned: (a) The values for non-protein nitrogen tend towards a higher level (30–45 mg.) than those given by previous workers. (b) The normal range of urea nitrogen should also be considered higher (15–25 mg.) than previously reported by many workers. In this respect our results confirm McLean's.[43] (c) Uric acid may reach as high as 3.0 mg. in 100 cc. of blood in many normal individuals. (d) Surprisingly low figures were consistently obtained for creatinine (0.1 mg. or less in 100 cc. of blood). Any value above 1.0 mg. should be considered as possibly indicating a pathological condition. (e) Cholesterol values of normal blood were obtained with a micro-colorimetric method.

4. The method for the determination of non-protein nitrogen was modified so as to reduce greatly the time necessary for its performance.

5. A method is described for determining fatty substances and cholesterol in the same 5 cc. portion of blood.

6. A cell was devised for measuring the conductivity of the circulating blood.

We take this opportunity to express our appreciation to Dr. Charles Norris, Director of Laboratories, for his interest in this work. For valuable assistance in many of these determinations we thank Mr. Ernest Pauli. To the students of Bellevue Medical College and others who volunteered blood for this study, we also take pleasure in extending our thanks.

[42] Butterfield, E. E., *Am. J. Med. Sc.*, 1916, cli, 63.

[43] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 223.

COMPOSITE TABLE.

No.	Sex	Name.	Age.	Weight.	Wassermann test.*	Red count.	White count.	Corpuscular volume.	Hemoglobin.	Solids.	Total blood N.	Total plasma N.	Total serum N.	Mg. per 100 cc. of blood.											Blood conductivity.	Serum conductivity.	H ion P_H (blood).	CO_2 bound by plasma.	Specific gravity, serum.	Freezing point depression.	Refraction Δ $Nd \times 10^5$.
														Non-protein N.	Urea N.	Ammonia N.	Uric acid.	Creatine.	Creatinine.†	Residual N.	Blood sugar.	Blood fat.	Cholesterol.	Blood chlorides.	Plasma chlorides.						
			yrs.	*lbs.*		*millions*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*													Λ	Λ		*per cent*		°C.	
1	♂	E. B.	17	108	—	3.8	7,000	37	80	21.1	3.1	1.25	1.28	47	26	0.7	4.0	3.9	0.1	17.6	52	110.0	34		637	55.5	117.1	7.56	59.4	1.0295	0.570	16.4
2	♂	M. B.‡	19	138	—	4.8	4,000	43	100	22.5	4.1	1.40	1.43	38	20	0.7	1.5	3.0	0.1	15.8	76		57	490	612	45.0	115.0	7.54	66.1	1.029	0.560	18.9
3	♂	F. R.	20	135	+++	4.3	9,000	46	86	22.4	3.6	1.28	1.37	37	19	0.5	1.8	4.9	0.1	15.1	85	79.4	45	480	580	48.5	117.4	7.60	65.2	1.0292	0.557	17.5
4	♂	F. R.§	20	135	+++	4.9	10,000	47	85	20.0	3.3	1.30	1.24	35	18	0.4	2.5	5.1	0.1	14.0	60	88.1	33	531	631	52.0	119.7	7.58	59.4	1.0297	0.561	17.6
5	♂	I. C.	20	144	—	5.0	7,800	49	90	22.1	3.6	1.25	1.35	32	22	0.5	3.1	5.7	0.1	6.6	133	66.4	38	480	600	48.4	117.6	7.52	59.4	1.0292	0.575	17.8
6	♂	H. N.	20	152	++	5.3	7,000	46	83	24.6	3.7	1.30	1.31	32	19	0.4	0.7	3.8	0.1	11.2	100	126.4	61	450	615	46.7	119.8	7.54	64.2	1.0294	0.595	18.0
7	♂	B. R.	21	181	—	5.0	8,500	49	100	22.1	3.6	1.25	1.27	34	17	0.4	1.2	4.3	0.1	15.2	76	115.5	48	450	605	46.3	121.5	7.58	62.3	1.0267		17.4
8	♂	E. A.	21	158	—	5.0	6,500	43	90	23.3	3.5	1.29	1.30	33	15	0.5	3.1	3.7	0.1	15.1	64	93.8	33	380	590	47.9	122.9	7.60	67.1	1.0275	0.576	16.9
9	♂	E. T.	21	192	—	5.6	6,000	48	90	20.9	3.2	1.37	1.21	35	17	0.4	2.4	5.6	0.4	14.7	100	109.7	52	510	618	46.3	124.5	7.60	67.1	1.0265	0.570	16.5
10	♂	L. D.	21	143	—	4.2	6,000	48	79	22.5	3.5	1.33	1.32	35	21	0.4	2.5	5.5	0.1	10.5	69		43	400	560	43.9	116.3	7.52	60.3	1.0278	0.567	17.4
11	♂	M. S.	22	165	—	5.0	7,000	43	82	23.4	3.6	1.17	1.20	32	19	0.5	1.6	5.4	0.1	9.8	85	90.3	48	460	618	47.9	119.8	7.56	61.3	1.0274	0.577	
12	♂	A. L.	22	160	—	5.0	11,000	50	85	23.7	3.7	1.25	1.38	44	23	0.5	1.8	6.3	0.5	17.5	60	66.0	41	400	525	44.0	117.6	7.54	69.0	1.0287		17.8
13	♂	L. F.	22	183	—	4.4	5,000	42	75	21.9	3.3	1.18	1.32	34	21	0.4	1.0	4.4	0.1	11.4	90	115.5		480	620	54.6	121.1	7.55	62.3	1.0278	0.570	16.8
14	♂	M. R.	23	158	—	5.5	6,000	49	90	21.5	3.4	1.40	1.30	39	20	0.5	2.3	4.3	0.1	14.1	80	98.2	58	460	575	56.8	113.4	7.58	77.8	1.0278	0.558	17.3
15	♂	W. Mc.	23	165	—	5.3	7,000	53	105	23.2	3.9	1.30	1.40	35	19	0.6	2.7	4.7	0.1	12.1	83		53	500	615	41.8	122.1	7.50	62.3	1.0295		17.8
16	♂	R. T.	25	131	—	5.0	5,000	49	70	24.9	3.6	1.31	1.30	33	16	0.7	3.6	3.9	0.1	13.6	84	119.0	41	440	560	47.5	118.1	7.56	64.2	1.0280	0.564	17.5
17	♀	J. M.	26	139	—	4.3	4,500	40	90	23.7		1.28	1.20	32	16	0.4	1.3	6.1	0.1	12.7	68		44	450	600	37.7	121.5	7.53	59.4	1.0258	0.568	15.8
18	♂	J. C.	26	220	+++	5.0	11,000	50	98	22.7	3.6	1.40	1.35	47	25	0.6	2.9	5.2	0.1	18.7	124	114.0	38		600	47.5	116.2	7.55	67.1	1.0298	0.565	18.2
19	♂	W. B.	28	184	—	5.0	6,000	45	100	21.6	3.7	1.30	1.30	36	22	0.4	3.3	2.7	0.1	11.4	87	97.5	39	490	610	53.7	121.5	7.53	68.1	1.0286	0.561	17.3
20	♂	W. B.§	28	184	—	5.0	6,000	46	100	23.0	3.5	1.30	1.26	34	17	0.5	3.0	2.5	0.1	14.9	50	102.1	34	475	637	52.1	122.4	7.59	55.5	1.0293	0.578	17.0
21	♀	D. Mc.	30	115	?			42	82		3.0	1.19	1.41	40	21	0.4	1.3	2.6	0.1	17.5	122	320.0	24		620		115.4	7.59	56.5	1.0380	0.582	18.9
22	♀	H. S.	35	130	?			43	85		3.0	1.10	1.35	43	18	0.5	0.3	3.4	0.1	22.6	119	187.0	43		575		122.4	7.57	58.4	1.0370	0.600	16.2
23	♂	L. L.‖	38	136	—	5.2	6,500	50	90	21.6	3.5	1.20	1.30	27	17	0.4	3.3	4.7	0.1	7.4	250‖	158.8		450	595	41.6	116.6	7.52	59.4	1.0299	0.710‖	18.1
24	♂	F. F.	39	220	—	4.8	10,000	43	80	24.1	3.4	1.30	1.40	41	10	1.1	3.2	3.8	0.1	27.9	123	112.0	47		600		117.0	7.56	64.2	1.0301	0.577	18.4
25	♂	A. K.	45	118	—	3.8	11,000	38	75	21.8	3.2	1.40	1.40	52	23	0.7	3.5	4.8	0.8	24.2	100	128.0	24		637	47.2	121.6	7.55	58.4	1.0295	0.605	16.6
26	♀	S. L.	45	145	—			46	95	21.5	3.4	1.20	1.20	28	10	0.4	0.6	5.0	0.1	16.1	57			368	500		120.8	7.59	57.4	1.0283	0.550	16.3
27	♀	O. B.	48	124	—	4.3	7,000	39	92	21.7	3.2	1.30	1.32	40	22	0.4	1.1	3.0	0.1	16.5	120	197.0	23		575	47.2	113.8	7.58	66.1	1.0307	0.577	18.8
28	♀	E. W.	50	145	—	3.8	6,000	33	75	20.1	3.1	1.20	1.20	38	17	0.4	0.6	3.0	0.1	19.2	115	149.0	19		525	64.0	120.1	7.59	68.1	1.0284	0.562	16.2
29	♂	G. B.	55	180	—	4.5	8,000	43	82	21.6	3.3	1.20	1.30	45	16	0.7	0.8	5.9	0.4	25.9	100	108.0	17		525	47.0	112.0	7.55	65.2	1.0241		17.0
30	♀	M. L.	59	160	?	4.0	9,000	39	80	23.5	3.3	1.20	1.30	45	17	0.4	1.8	5.1	0.1	24.9	72	145.0	23			49.8	115.4	7.58	62.3	1.0352	0.558	18.1

* Wassermann test (cholesterol antigen).

† 0.1 means 0.1 or less.

‡ Taken before breakfast.

§ 3 weeks later on same subject.

‖ Previously unrecognized diabetes.

(Gettler and Baker: Chemical and Physical Analysis of Blood in Thirty Cases.)

SOME OBSERVATIONS ON THE TETANY OF PARATHYROIDECTOMIZED DOGS.

By ISIDOR GREENWALD.

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(Received for publication, April 7, 1916.)

In a previous communication, the author[1] called attention to the marked retention of phosphorus after parathyroidectomy in dogs. Later experiments[2] showed that this retention was followed or accompanied, but never preceded, by a retention of sodium and potassium. The amount of phosphorus in the blood and serum was found to be increased and this increase was chiefly, if not entirely, in those compounds of phosphorus which are insoluble in the usual lipoid solvents but which are soluble in a mixture of dilute acetic, or hydrochloric, and picric acids. As this retention of phosphorus appears to be the most marked change in metabolism as yet observed after parathyroidectomy, it seemed desirable to investigate its significance further.

From the experiments of Gamgee, Priestly, and Larmuth[3] it is quite evident that if the phosphorus were retained as *o*-phosphate it could not be regarded as the cause of tetany. Experiments in this laboratory[4] confirmed the results of Gamgee, Priestly, and Larmuth as to the non-toxicity of relatively large amounts of sodium phosphate. It was found possible to increase the amount of acid-soluble phosphorus to twenty times the normal amount without marked effects on the behavior of the animals. Other phosphorus compounds were then sought as the possible toxic agents. Attention was immediately directed to inosinic acid. This substance, which is present in muscle tissue in con-

[1] Greenwald, I., *Am. J. Physiol.*, 1911, xxviii, 103.

[2] Greenwald, *J. Biol. Chem.*, 1913, xiv, 363, 369.

[3] Gamgee, A., Priestly, J., and Larmuth, L., *J. Anat. and Physiol.*, 1877, xi, 255.

[4] Greenwald, *J. Pharm. and Exp. Therap.*, 1915, vii, 57.

siderable quantity is, chemically, one of the simplest of the nucleic acids. The large amount present in a tissue so poor in nuclei as is muscle, and its ready extraction therefrom, indicate that, physiologically, it is something quite different. Besides phosphoric acid, it yields upon hydrolysis, a pentose, *d*-ribose, and hypoxanthine. The latter, according to Schmiedeberg,[5] produces convulsions in frogs. Moreover, the author had observed the effects of intravenous injections of xanthine in dogs and had been struck by their resemblance to the tetany of parathyroidectomized dogs. It was therefore determined to investigate the possible rôle of inosinic acid in the production of tetany.

Barium inosinate was prepared by the method of Haiser and Wenzel.[6] It was found that when added to horse serum, both the phosphorus and the hypoxanthine could be quantitatively recovered from the filtrate obtained by treating the serum with a saturated solution of picric acid in 1 per cent acetic acid. The phosphorus was determined by the method of Pouget and Chouchak as adapted by the author[7] to the estimation of phosphorus in serum. For the determination of the purine nitrogen, larger amounts of the picric acid filtrate were evaporated on the water bath to a small volume. The entire mass, including the crystallized picric acid, was transferred to an apparatus for the continuous extraction of liquids and there treated with benzene until no more picric acid was removed. About 5 per cent, by volume, of concentrated sulfuric acid was then added and extraction continued. After this was apparently complete the aqueous solution was heated to drive off the residual benzene, cooled, made alkaline with ammonium hydroxide, and treated with ammoniacal silver solution. No precipitate was obtained from the equivalent of 225 cc. of normal horse serum, but if barium inosinate had been added a heavy white precipitate appeared. This was filtered off, washed with dilute ammonium hydroxide, and decomposed with hydrochloric acid. The silver chloride was filtered out, the filtrate neutralized with sodium hydroxide, made acid with acetic acid, and the hypoxanthine reprecipitated in the usual manner

[5] Schmiedeberg, O., *Ber. chem. Ges.*, 1901, xxxiv, 2552.

[6] Haiser, F., and Wenzel, F., cited by Steudel, H., *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, ii, 602.

[7] Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

with copper sulfate and sodium bisulfite. The precipitate was filtered out, washed with hot water, and its nitrogen content determined. The amount found corresponded with the amount of barium inosinate added.

When this method was applied to dog serum it was found that neither normal serum nor that of parathyroidectomized dogs in which tetany was just developing contained any purine nitrogen. In two experiments, in which the serum was not absolutely clear, a trace of purine nitrogen was obtained, but this may have been due to the nuclear material of the cells.

In order to test further the possibility of inosinic acid being the toxic agent in tetany, a solution of sodium inosinate, prepared from the barium salt and sodium sulfate, was injected intravenously into a dog from which the thyroid and parathyroids had been removed 5 hours previously. No effect was observed, although the amount used was greater than could have been tolerated if inosinic acid were the toxic agent in tetany. Determinations made after the injection of the sodium inosinate and also when tetany appeared on the following day showed that at the latter time the amount of acid-soluble phosphorus in the serum was less than in the former instance.

The hypothesis that inosinic acid is the toxic agent in the causation of the tetany of parathyroidectomized dogs must therefore be abandoned and the significance of the retention of phosphorus remains, for the present, unexplained.

Recovery of the Phosphorus of Barium Inosinate Added to Serum.

	mg.	mg.	mg.
Acid-soluble phosphorus in 1 cc. of serum.	0.063	0.061	0.018
Phosphorus in added barium inosinate....	0.058	0.049	0.013
Total.....	0.121	0.110	0.031
Found.....	0.117	0.116	0.031

Recovery of the Nitrogen of Barium Inosinate Added to Serum.

	mg.	mg.
Nitrogen of the barium inosinate added to 250 cc. of serum.....	22.09	24.07
Purine nitrogen found.....	19.45	23.47

Experiment Showing the Effect of the Intravenous Injection of Sodium Inosinate.

To a solution of 1.8345 gm. of barium inosinate in 100 cc. of hot water there was added 0.460 gm. of dry sodium sulfate dissolved in water. After standing over night, the mixture was filtered and the filtrate and washings were diluted to 150 cc. The solution contained no barium and only a trace of sulfate. The liquid was then made approximately isotonic with blood by the addition of 0.60 gm. sodium chloride.

Dec. 11, 1914. Dog weighing 4.18 kilos. Parathyroidectomy at 11 a.m. Between 4.00 and 4.27 p.m., injected 145 cc. of the above solution into a femoral vein, using cocaine anesthesia. No symptoms. 4.43 p.m., drew a small sample of blood. Serum obtained contained 0.127 mg. acid-soluble phosphorus in 1 cc.

Dec. 12, 5 p.m. Fairly well marked twitching. Bled to death under cocaine anesthesia. The serum obtained from the first few cc. of blood contained 0.097 mg. acid-soluble phosphorus in 1 cc.

THE FORMATION OF SPECIFIC PROTEOCLASTIC FERMENTS IN RESPONSE TO INTRODUCTION OF PLACENTA.

By FLORENCE HULTON.

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(Received for publication, May 4, 1916.)

The digestive power on pure proteins of the serum of injected rabbits was reported in a previous paper.[1] These results showed plainly "that under the conditions of the experiment there is practically no digestion with the blood of the injected animal in excess of that which takes place with the serum of the normal control animal." There were two other points that it seemed wise to investigate: (1) the digestive action on placental protein of the serum of an animal injected with placenta; (2) the digestive action of this same serum on various pure proteins.

Two dogs were given three injections of a placental emulsion, a week apart. The placenta used was human placenta, procured soon after delivery. All fetal remnants were carefully dissected away from the placenta, immediately after delivery with sterile instruments, and the upper and lower surfaces cut off. It was then wrapped in sterile gauze and placed in a desiccator over chloroform. As soon as it was received in the laboratory, it was ground in a sterile grinder, caught in a sterile dish, extracted with Ringer's solution, and strained through sterile gauze. The first two injections were 10 cc. each, the first given intraperitoneally, the second intramuscularly. The third injection of 20 cc. was administered 10 cc. intraperitoneally, and 10 cc. intramuscularly. The following day the dogs were etherized and bled to death from the carotid, the blood being caught in a beaker and stirred constantly to defibrinate. Two normal dogs were bled in the same manner.

[1] Hulton, F., *J. Biol. Chem.*, 1916, xxv, 163.

The defibrinated blood was centrifuged, the serum decanted off, and portions were used to determine non-protein nitrogen, removing the protein according to the method of Folin,[2] using absolute ethyl alcohol instead of methyl alcohol. Check analyses were done with trichloroacetic acid according to Greenwald.[3] Since the two methods checked so closely, either method was used with the later analyses.

The rest of the serum was divided into portions, 10 cc. in the case of the placental protein, and 5 cc. in the case of the other proteins. The serum was placed in a flask with 1 gm. of the particular protein, made alkaline with 2 cc. of 0.1 N NaOH, diluted up to 50 cc. with Ringer's solution, covered with 5 cc. of toluene, and incubated for 48 hours at 37.5°C. After incubation, the contents of the flasks were mixed with nine volumes of absolute ethyl alcohol and allowed to stand 24 hours. The filtrate was treated with saturated $ZnCl_2$ solution. Gliadin in all cases was removed with trichloroacetic acid.

The results are summarized in the tables.

TABLE I.

Digestion of Proteins by Blood Serum.

Protein.	Non-protein N in 100 cc.			
	Serum of normal dogs.		Serum of dogs previously injected with saline emulsions of human placenta.	
	Before	After	Before	After
	Incubation with protein.		Incubation with protein.	
	mg.	*mg.*	*mg.*	*mg.*
Placental protein.....	24	65	33	52
	23	62	30	58
Protamine.....	24	193	30	253
Casein.....	24	145	30	88
Bence-Jones protein..	24	75	30	66
Phaseolin.....	24	74	30	88
Edestin.....	24	55	30	55
Gliadin.....	24	66	30	155
Soy bean globulin.....	24	54	30	56
Milk albumin.....	24	46	30	55

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

[3] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE II.

Increase of Non-Protein N in 100 Cc. of Serum.

Digestion with	Injected animals.*	Normal animals.
	mg.	*mg.*
Placental protein	19	41
	28	39
Protamine	223	169
Casein	58	121
Bence-Jones protein	33	51
Phaseolin	55	50
Edestin	25	31
Gliadin	125	42
Soy bean globulin	26	30
Milk albumin	25	22

* Injected animals in each case were injected with human placenta emulsion.

The results show that, under the conditions of the experiment, with placental protein there is no increase in the digestive powers of the serum of the injected animal, that of the normal animal showing a slightly greater rise in non-protein N. The greatest amount of digestion with pure proteins has taken place with protamine. This might be explained on the ground that protamine, being one of the simpler proteins, is more readily split into its amino-acid components. Gliadin is split to a marked degree by the blood of the injected animal.

The figures indicate that the blood of the dog possesses to a marked degree proteoclastic activity, which is not increased toward either placental protein or the other proteins used in the experiment by injecting placental proteins. This activity may be due to ferments present as normal constituents of the blood or appearing there under certain metabolic conditions, just as trypsin appears in the urine. Any specific reaction in pregnancy or in neoplastic conditions it would seem could hardly be due to increased ferment production in the blood and the results do not bear out the statement that placental proteins in the circulating blood call forth a protective enzyme which by digestion removes the placenta from the blood.

SUMMARY.

Placental protein is not digested to any greater degree by the serum of an animal sensitized to placenta than by the normal serum.

The digestive power of the serum of an animal sensitized to placenta is not increased for casein, Bence-Jones protein, phaseolin, edestin, soy bean globulin, or milk albumin. Casein is digested to a marked degree by the normal serum, and in most cases the normal serum possesses the more marked activity.

Protamine is digested to a marked degree in both cases, the injected animal showing increased activity.

Gliadin is not digested to any great extent by the normal serum, but is by the serum of the injected animal.

In general it may be said that the injection of placenta does not increase the general or call forth the specific proteoclastic ferment of the blood.

I take this opportunity to thank Dr. A. E. Taylor, under whom the work was begun, for his interest and help, and also Dr. M. M. Peet, of the Department of Research Surgery, for his help in bleeding the normal dogs.

A COLORIMETRIC METHOD FOR THE ESTIMATION OF FREE FORMALDEHYDE AND HEXAMETHYLENAMINE.

By R. J. COLLINS and P. J. HANZLIK.

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(Received for publication, April 24, 1916.)

It has previously been ascertained[1] that the phloroglucinol or Jorissen test is among the most sensitive color tests for free formaldehyde. The advantages claimed for it are: (1) simplicity; (2) great sensitivity; (3) it reacts with free or liberated formaldehyde only, and does not itself liberate formaldehyde from such compounds as hexamethylenamine; (4) finally, with the phloroglucinol test there is a gradation of colors, from pink to deep red, depending on the concentration of formaldehyde, which suggested to us the possibility of its application in a quantitative manner. However, the colors are not permanent. Their maximum intensity is reached within 3 minutes and then they gradually change to a violet and finally disappear altogether. This is particularly true with the high dilutions. Therefore we sought for a substance, or mixture of substances, which would give us the same quality of color as in the phloroglucinol test, and yet be permanent when used in making a set of standards.

After considerable experimentation with metallic salts and different dyes, a mixture of Congo red (0.025 per cent in water containing 5 per cent alcohol) and methyl orange (0.01 per cent in water) was found to be satisfactory. A mixture of these in the proper proportions is only necessary in matching the reds of phloroglucinol in the lower concentrations of formaldehyde. For the higher concentrations Congo red alone suffices.

[1] Hanzlik, P. J., and Collins, R. J., *Arch. Int. Med.*, 1913, xii, 578. The reagent consists of phloroglucinol (reagent-Merck) 0.1 gm. dissolved in 10 cc. of 10 per cent sodium hydroxide.

It is important to note that samples of Congo red, even of the same manufacture, are apt to differ considerably in point of quantity, but apparently not in quality, of color. Owing to this it is necessary to standardize any particular specimen of Congo against a known standard made from an inorganic salt before it can be used to prepare the permanent Congo standard for the dilutions. We believe that we have successfully achieved this with potassium bichromate and sulfuric acid. The following procedure for standardizing Congos has been found to work satisfactorily.

1.7616 gm. of $K_2Cr_2O_7$ by titration against alkali (roughly about 30 cc. of a 5 per cent solution) and 11.5537 gm. of absolute H_2SO_4 (about 7 cc. concentrated) are mixed and diluted to the mark in a 50 cc. Nessler tube with a column of 12 cm. This is equivalent to 50 cc. of 1: 100,000 absolute formaldehyde, or 50 cc. of a Congo standard 1: 100,000, which contains 0.000625 gm. of the original dry Congo red, or 2.5 cc. of 0.025 per cent. It is only necessary, therefore, to prepare a proper mixture of $K_2Cr_2O_7$ and H_2SO_4, and when an unknown Congo solution is standardized against this, the quantity used will contain 0.000625 gm. of Congo. The strong solution can then be diluted or made accordingly, the standard dilutions from this to be equivalent to the different concentrations of formaldehyde.

The proportions of Congo red and methyl orange which have been worked out for the different concentrations of absolute formaldehyde are presented in Table I. The basic solutions for making the standards are not mixed until ready for use. We have kept both solutions for 2 to 3 months without any demonstrable change. However, after the dyes are mixed and diluted to the proper volume with water, deterioration occurs with the weaker colors within a week, the stronger colors remaining permanent for at least 2 weeks. It is advisable, therefore, to prepare a set of dilutions each day. The technique of making standards consists simply in measuring the quantity of each solution necessary with an accurately graduated pipette into 50 cc. Nessler tubes of the short variety with columns of fluid 12 cm. high. These are then diluted with water to the mark, gently agitated, and are ready for use.

For the estimation of formaldehyde in clear aqueous solutions

containing formaldehyde, the technique is as follows: An aliquot portion, 1, 5, or 10 cc., of the solution are measured into the Nessler tube, phloroglucinol reagent (1 to 2 cc.) is added, and the whole is diluted to the 50 cc. mark and gently agitated. After standing 3 minutes the tube is matched against a series of tubes containing the standard colors just described. The results can be expressed in percentage or gm. of formaldehyde. In calculation allowance must be made for dilution in the Nessler tube.

TABLE I.

Standard Mixtures of Congo Red and Methyl Orange.

Concentration of formaldehyde.*	Percentage concentration of formaldehyde.	Congo red** 0.025 per cent.	Methyl orange** 0.01 per cent.
		cc.	*cc.*
1: 20,000	0.005	20.0	0
1: 30,000	0.0033	11.0	0
1: 40,000	0.0025	9.0	0
1: 50,000	0.002	8.0	0
1: 60,000	0.0016	5.0	0
1: 80,000	0.00125	4.0	0
1: 100,000	0.0010	2.5	0
1: 200,000	0.0005	0.85	0.40
1: 250,000	0.0004	0.65	0.35
1: 500,000	0.0002	0.25	0.18
1: 750,000	0.00014	0.20	0.15
1: 1,000,000	0.00010	0.13	0.10

* Total volume of solution = 50 cc. in a Nessler tube with a 12 cm. column.

** The quantities of Congo red and methyl orange here indicated are mixed and diluted with water to the mark in a 50 cc. Nessler tube with a 12 cm. column.

For instance, if 5 cc. of the formaldehyde solution were used and diluted to 50 cc., then the percentage concentration to which the matched color corresponds is multiplied by 10. This gives the percentage concentration of the formaldehyde solution. From this the absolute weight in gm. of formaldehyde can, of course, be easily calculated.

For urine the procedure must be slightly modified because of the presence of phosphates which, when precipitated by the alkali of the phloroglucinol reagent, interfere with the reading of

the color. These are removed by adding to an aliquot portion of the urine a few drops of concentrated (50 per cent) sodium hydroxide and filtering, then washing with a little water to the original volume.

If the urine is concentrated or deeply colored, an equal volume of the same or some other urine of about the same color must be added to the standards containing the mixtures of Congo red and methyl orange. The estimation is then carried out in exactly the same way as for aqueous solutions.

We have compared the colorimetric method with the Romijn or iodine method,[2] the U.S.P. peroxide method with heat,[3] and the sodium hydroxide heat pressure method.[4] Distillates from known quantities of hexamethylenamine were used. Results illustrative of the data obtained are indicated in Table II. The following mean percentage recoveries of the theoretical yield of formaldehyde were obtained with the different methods: colorimetric, 99 per cent; Romijn, 88 per cent; U.S.P., extremely variable (none to 96 per cent); hydroxide and pressure, extremely variable (none to 90 per cent). In order to be able to obtain these percentage recoveries with the other methods usually ten times the quantity of the distillate was necessary for single estimations, as compared with the colorimetric. It was also found that the colorimetric method is more accurate with higher dilutions of formaldehyde, because it is more difficult to read small differences between more intense than between weaker colors.

For direct application to urine the other methods with which the colorimetric was compared are not suitable, since urine itself consumes iodine, hydroxide, and peroxide. Distillation of urine containing such an easily decomposable formaldehyde compound as hexamethylenamine is not permissible if an idea of the formaldehyde liberated during its passage through the body is to be obtained. Here the only choice is the colorimetric method.

Salkowski[5] has recently practiced a modification of the Leach ferric chloride test as a colorimetric method for the estimation of formaldehyde.

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[2] Sutton, F., Volumetric Analysis, Philadelphia, 9th edition, 1904, 370.

[3] U. S. Pharm., 8th revision, 1900, 266.

[4] Smith, C. E., *Am. J. Pharm.*, 1898, lxx, 86.

[5] Salkowski, E., *Biochem. Z.*, 1915, lxviii, 337; 1915, lxxi, 365.

In this hydrochloric acid is used, and this precludes its use for the estimation of free formaldehyde in the presence of easily decomposable formaldehyde compounds, such as hexamethylenamine. We have not practiced the phenylhydrazine-nitro-prusside test for quantitative purposes because of difficulties with the test which were pointed out in a previous paper. It has been used by Dunning.[6]

TABLE II.

Estimation of Formaldehyde in Hexamethylenamine.

Amount of hexamethylenamine.	Equivalent in HCHO.	Formaldehyde recovered.			
		Romijn.	U. S. P.	Alkali.	Colorimetric.
gm.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*
1.0	1.28	51(4)* 75(40)	None(4)		98(4)
0.5	0.64	53(7.8) 76(78)	32(7.8) 86(78)	41(7.8) 90(78)	100(7.8)
0.05	0.064	82(25) 96(100)	87(25) 96(100)	90(25)	96(8.2) 98(8)
0.005	0.0064	None(22) 96(220)	None(22) " (100)	None(22) " (100)	100(7.8) 100(22) 102(15)

Hexamethylenamine in each case above was decomposed with the aid of weak acid (three drops of 85 per cent phosphoric).

1.0	1.28	None(1) 78(10)	None(1)	None(1) 82(10)	78.1(1)
0.5	0.64	None(1) 74.25	None(1) 69(20) 93(25)	None(1) 78(20)	78.1(1)
0.05	0.064	74.7(25) 98(100)	76(100)		78.1(25)
0.005	0.0064	None(25) 48(50)	None(25)	None(25)	78.1(25)

Hexamethylenamine in each case was decomposed with the aid of strong acid (20 cc.), sometimes phosphoric, sometimes sulfuric.

* The figures in brackets denote the number of cc. of distillate used for estimation. The distillate in each case measured 1,000 cc.

[6] Dunning, H. A. B., *J. Am. Pharm. Assn.*, 1914, iii, 637.

Estimation of Hexamethylenamine.

For this it is only necessary to distil an aliquot portion of the solution or urine containing hexamethylenamine, and then apply the colorimetric procedure described above for free formaldehyde to the distillate (distilled or diluted to a definite volume). Distillation is not facilitated by the addition of acid. On the contrary, there seems to be a loss when large or moderate quantities of phosphoric or sulfuric acid are used. Only 78 per cent was recovered (see Table II), as compared with 96 to 100 per cent when only a trace or no acid was used. For calculating the amount of hexamethylenamine recovered the formaldehyde determined is divided by 1.28, since 1 gm. of hexamethylenetetramine yields 1.28 gm. of formaldehyde.

In order to arrive at the proper amount of undecomposed hexamethylenamine due allowance must be made for free formaldehyde if any is present, by subtracting this from the total amount of formaldehyde recovered in the distillate. This is of importance in connection with urines and old standing solutions of hexamethylenamine.

That a good recovery is obtained with this method is indicated by the results presented in Table II, which when converted into hexamethylenamine will indicate the same percentage recovery as the formaldehyde.

The administration of 2 gm. of hexamethylenamine by mouth, on two different occasions, to the same individual, resulted in a recovery of 38 per cent in 6 hours on one occasion, and 71 per cent on another occasion when the excretion was completed. During both experiments the water intake was maintained constant. 1 gm. of the drug taken on two different occasions by the same individual gave recoveries of 70 and 76 per cent, respectively. In these two experiments no attention was paid to the fluid intake; and in all cases (except the 6 hour experiment) the urine was collected until it failed to give positive tests with bromine water and the phloroglucinol reagent. Similar results were recently reported by Falk and Sugiura,[7] using their iodine method for the quantitative estimation of hexamethylenamine. Owing to

[7] Falk, K. G., and Sugiura, K., *J. Pharm. and Exp. Therap.*, 1916, viii, 39.

the few observations which we have made, no explanation for these low excretory results can as yet be offered. Further observations on this and other features of hexamethylenamine excretion and decomposition will be reported later.

CONCLUSIONS.

1. A method for the colorimetric estimation of free formaldehyde and hexamethylenamine is described, in which the phloroglucinol reagent is used.

2. Permanent color standards which exactly match the phloroglucinol in different concentrations of formaldehyde can be made from mixtures of 0.025 per cent Congo red and 0.01 per cent methyl orange.

3. These mixtures have been experimentally determined for a number of concentrations of formaldehyde between 1: 1,000,000 and 1: 20,000.

4. The colorimetric method is more accurate than the Romijn, U.S.P., and hydroxide pressure methods.

5. It is more accurate for high than for low dilutions of formaldehyde.

6. The colorimetric method possesses an important advantage over other methods in that it is directly applicable to urine for the determination of free formaldehyde.

FURTHER STUDIES ON THE NUTRITIVE DEFICIENCIES OF WHEAT AND GRAIN MIXTURES AND THE PATHOLOGICAL CONDITIONS PRODUCED IN SWINE BY THEIR USE.*

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Plates 1 to 5.

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In earlier studies on the influence of restricted natural feeds on growth and reproduction it was observed that a ration from the wheat plant[1]—wheat grain plus wheat straw—was wholly inadequate with heifers for reproduction and in some instances for continued growth. With swine, confined to the wheat grain and a suitable salt mixture, growth soon ceased and the animals passed into a poor condition, while a corn and salt mixture ration was at least sufficient for slow growth and continued well-being.[2] Similar results are on record with rats, and only when a liberal supply of casein and fat-soluble A was added to a wheat grain and salt mixture was growth continuous and, in the case of rats, reproduction possible.

Previous work in this laboratory has shown the necessity of two hitherto unappreciated factors in the diet of the growing animal. One of these is associated with butter fat and certain other fats and is found also in relatively small amounts in the grains, but in considerably higher concentration in the leaves of certain plants, as alfalfa and cabbage, than in the grains. The

* Published with the permission of the Director of the Agricultural Experiment Station.

[1] Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull. 17*, 1911.

[2] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

other one is soluble in water and in alcohol and is the only one concerned in the production and cure of polyneuritis resulting from feeding fowls or pigeons an exclusive polished rice diet. This one appears never to be associated with fats. McCollum and Kennedy[3] have suggested the advisability of designating these two substances as fat-soluble A and water-soluble B, respectively, until we possess more knowledge of their chemical nature.

We have in these earlier papers expressed the view of the possibility of inherent toxicity in the wheat kernel, a view now made probable by the fact that the wheat embryo yields by ether extraction an oil of distinct toxicity and a residue far more innocuous than the embryo itself.[4] That this embryo carries a considerable quantity of a toxic substance is further evidenced by the fact that on increasing the mass of embryo in the rations of herbivora, earlier abortions are produced than when the ration carries the whole wheat grain only.[5] When the diet of swine consisted of whole wheat, wheat gluten plus a suitable salt mixture, the individual soon failed to grow and passed into a pathological condition. Loss of weight, rough coat, emaciation, lack of coordination of the muscles, labored breathing, and even blindness manifested themselves. Experiments with rats showed that with an abundance of better proteins from milk powder or casein, a more efficient salt mixture and the addition of fat-soluble A, and with whole wheat constituting approximately 65 per cent of the ration, the depressing action of this toxicity could be overcome.

With swine receiving similar additions to the wheat grain, but not in the same quantitative order, growth was normal but reproduction failed. It is important in this connection to call attention to the fact that when the additions to wheat were only salts and butter fat but without casein, the curve of growth was improved (see Chart V[3]), but ultimately these animals failed with symptoms similar to those shown on the wheat, salt mixture diet. It became evident as this work with swine progressed that we were not dealing in these cases with a deficiency in the diet, but that some inherent substance in the ration was either interfering

[3] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

[4] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.

[5] Unpublished data from this laboratory.

with the use of certain constituents necessary for perfect nutrition, or was directly causing nutritional disturbances.

It has been pointed out by Funk[6] and others that beri-beri is a deficiency disease, and it has been further shown by McCollum and Davis[7] that this deficiency probably lies in the absence of water-soluble B. In the work to be reported here evidence is presented tending to show that these pathological conditions manifested by swine are, as far as the histological picture of the spinal cord is concerned, analogous to, if not identical with the pathological condition recorded for polyneuritis in fowls, but here induced by an inherent toxicity and not by a deficiency.

Should our premises be correct, then it is apparent that there are several avenues by which similar pathological conditions of nutritional origin may be brought about. This is extremely important for purposes of a proper consideration of the causes of a number of diseases of nutrition, such as scurvy, rickets, pellagra, and beri-beri. McCollum has advanced the hypothesis that the factors for normal nutrition with purified food substances are a proper supply of proteins, energy, salts, fat-soluble A, and water-soluble B.[8] This hypothesis is demonstrable with purified foodstuffs, but in nutrition with natural food materials, besides the factors of poor proteins, poor salt mixture, absence or low supply of fat-soluble A, or water-soluble B there arises the possibility of the presence of substances exerting a toxic action. For that reason it becomes apparent that *for normal nutrition with natural foodstuffs not only must there be a plentiful supply and proper constitution of the above essential factors, but also a supply sufficient to overcome any inherent toxicity or else an absence of the toxicity itself.* Of course it is assumed that some kinds of toxicity of natural foodstuffs can be overcome by a plentiful supply of the normal factors of nutrition, but we do not hold that this is always possible.

EXPERIMENTAL.

Experiment 1.—All the experiments described in this paper were made with female swine strictly confined to pens. In Text-fig. 1 are presented the

[6] Funk, C., *J. Physiol.*, 1911, xliii, 395.

[7] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

[8] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 231.

growth curves of two animals receiving Ration 1, consisting of 95.5 pounds of wheat meal, 2.5 pounds of wheat gluten, 2 pounds of butter fat, 323 gm. K_2HPO_4, and 513 gm. Ca lactate. The salt mixture is one which when added to a mixture of 70 parts of corn meal and 30 parts of gluten feed was adequate for a fair rate of growth and perfect maintenance. The protein content of the ration was approximately 13 per cent. Symptoms of malnutrition and failure to grow appeared only after a lapse of 9 months, and then loss of weight set in, followed by difficulty in locomotion, rough coat, labored breathing, and muscular twitching. Fig. 1 shows the appearance of one of these animals before the last stages. At this time this animal could still walk, although with difficulty, but there was not the extreme prostration appearing in other animals on similar diets and manifested by walking on the knees or dragging the rear quarters.

The animal was killed and the suprarenals, thyroid, parathyroid, spinal cord, and brain were removed. There were no certain abnormal conditions noted in the ductless glands. The spinal cord, which was examined by Dr. Miller, did show marked histological changes and the studies recorded for this animal, as well as others described below, are confined to the spinal cord. The vertebral canal contained a considerable quantity of fluid and the motor cells of the spinal cord exhibited marked alterations. Fig. 3 is a microphotograph of this pathological cord from the lumbar region. Fig. 2 shows a section of a normal pig's spinal cord in the same region and taken from an animal of approximately the same age as that of the pathological specimen. It will be noticed that there are marked differences in these specimens, although both were carried simultaneously through the same fixing and staining baths.[9]

In the normal cord the motor cells are not compressed and shrunken and are not surrounded by any appreciable quantity of fluid. The processes are intact, the nucleus and nucleolus remain distinct, and the Nissl granules likewise are distinct and separate. In the pathological cord we see a picture of marked contrast. The motor cells, surrounded by fluid, are compressed, the processes partly degenerated, the nucleus is shrunken, and the whole cell content stained as a uniform blue mass (black in photo).

[9] Blocks about 1 cm. thick were taken from the various regions of the spinal cord and throughout the series were treated as follows:

1. Fixed in Van Gehuchten's fluid for 8 hours.
2. Treated with alcohol of gradually increasing strength.
3. Cleared in a mixture of equal parts of oil of cloves and turpentine.
4. Embedded in paraffin.
5. Sections cut 5 and 7.5 μ thick.
6. Stained with erythrosin and toluidine blue.
7. Mounted in balsam.

These pictures of the cord changes are similar to those described by Vedder and Clark[10] for polyneuritis in fowls, but here produced in the presence of all known essentials for adequate nutrition. It would appear, therefore, that the inherent toxicity of the wheat kernel must be held directly responsible for these changes. The only possible criticism that could be raised against this conclusion is the factor of the quantity of fat-soluble A introduced into the diet. While 2 per cent of fat-soluble A (2 per cent of butter fat) is inadequate for normal growth of rats on a purified food diet, yet there is no evidence that this amount would not allow slow growth and continued maintenance of well-being in the animal. Further, in the diet here used there was undoubtedly some fat-soluble A introduced with the whole wheat grain.[11] The quantity of water-soluble B introduced was also probably ample, since the embryo of the wheat grain constituted about 5 per cent of this ration and that proportion is known to carry enough of this material for continued and normal growth.[7]

The pathological changes in the cord correlate very readily with some of the external symptoms manifested by these animals, such as stiffness and lack of muscular coordination.

Experiment 2.—In Text-fig. 2 is shown the growth curve of two female animals receiving 3 pounds of whole milk daily per individual, and what wheat meal they would consume (Ration 2). The wheat meal varied with the age of the animals from 2 to 4 pounds per individual daily, and constituted about 90 per cent of the dry matter of the ration. The plane of protein intake was between 12 and 15 per cent, and the butter fat consumed daily approximated 45 gm. This would make the per cent of butter fat in the ration vary from 2 to 4 per cent, depending upon the variation in the wheat meal consumed with increasing age. The milk protein constituted from 20 to 40 per cent of the total protein intake, likewise depending upon whether the animal was consuming 2 or 4 pounds of the wheat meal.

Growth was rapid and normal for 4 to 5 months, at which time the rate of growth began to decline and finally actual loss of weight set in. At this time there was extreme stiffness, twitching of the muscles, and dragging of the rear quarters. After being helped to stand these animals would fall again to their knees, draw the hind legs toward the fore feet, and from this position roll to one side. Fig. 4 shows these individuals. Postmortem examination was made by Dr. Miller and revealed the probable causes of the condition. The suprarenals, thyroid, parathyroid, and brain were re-

[10] Vedder, E. B., and Clark, E., *Philippine J. Sc., B*, 1912, vii, 423.
[11] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 179.

moved. Grossly, the ductless glands showed no marked change. The brain was not sectioned. The spinal canal contained a considerable quantity of fluid and in the spinal cord again distinct changes had occurred (see Fig. 5). The motor cells were shrunken, surrounded by fluid, and appeared to be under pressure. The processes had degenerated, the Nissl granules were crowded together, and the nucleus and nucleolus nearly obliterated. It should be noticed in the microphotograph that some of the motor cells were apparently intact and not all of them in the same degree of degeneration. The histological picture here presented is very similar to that described for the animals on Ration 1. The ventrolateral and the ventromedial groups of cells in the anterior horn of the spinal column are chiefly affected. In other words, those cells that are most concerned in the formation of the motor root of the spinal cord showed greatest degeneration.

Apparently swine were somewhat more susceptible than rats to the toxic substance of wheat, and it may well be suspected that other species will vary in this respect. Unpublished data indicate that chickens are considerably less susceptible to a restricted wheat ration than any of the animals so far involved in these inquiries. It should again be emphasized that all the necessary factors for continued growth were present in this ration and the only explanation of these results must lie in the presence of toxicity. It was, however, possible that in Experiment 2 described above there was an inadequate supply of salts. The introduction of but 3 pounds of milk daily may have given an insufficient quantity of this factor, especially after the animals reached the higher weights of 150 pounds or more.

Experiment 3.—For the reason stated above, animals were started on Ration 3, composed of 95.5 pounds of wheat, 2.5 pounds of wheat gluten, 323 gm. K_2HPO_4, 513 gm. Ca lactate, and 3 pounds of whole milk per individual daily. The salt mixture is the same one used with success in experiments with corn grain. For 6 months growth was practically normal (see Text-fig. 3) but after that time decline set in and the animals suffered in nutrition as is indicated in Fig. 6. This figure shows one of the individuals toward the end of the experiment.

The gross external symptoms were similar to those previously described for other animals on excessive amounts of whole wheat. No microscopical examination of the cord of these animals was made, but we have no doubt that the picture would have been similar to that already described.

It is apparent from the results of the above experiments that too large a proportion of whole wheat in the ration will introduce

such a mass of toxic material as to render it impossible for this animal to complete its cycle of life, even in the presence of a considerable quantity of all known factors necessary for growth.

The question of the cause of this pathological condition may, however, be more complex than would seem evident from the above experiments. In those experiments described it is fairly clear that the result is due to the introduction of toxic material. In two additional experiments which we wish to present, the factors operative may be more complex and raise the question whether there are not several ways by which this pathological condition may be produced. In 1914 we published[2] among other data the curves of growth of swine receiving a ration of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, and 10 parts of oil meal, 323 gm. of K_2HPO_4, and 513 gm. of Ca lactate. With this ration, presumably carrying a suitable salt mixture, a fair quality of protein, but probably a rather low quantity of fat-soluble A and the toxicity of the wheat embryo of the middlings, growth was not sustained and the animals passed into a pathological condition. The factors operative in this experiment may have been toxicity augmented by too low a supply of fat-soluble A. Of course, the mass of toxicity in this ration was appreciably lower than in a ration carrying 90 per cent or more of whole wheat. The embryo of the wheat grain constitutes about 5 per cent of the grain, which would mean a content of 4 to 5 pounds of embryo in 100 pounds of the wheat ration described in Experiments 1, 2, and 3. In the mixed grain ration the content of embryo would be much less, probably not over 2 pounds in 100 pounds of the mixture, based on the fact that wheat middlings contain about 6 per cent of this material.

Experiment 4.—In this experiment the ration (Ration 4) consisted of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 10 parts of oil meal, and a small daily allowance of roots and alfalfa. The latter did not constitute over 5 per cent of the dry matter of the ration. In this ration toxicity of the embryo was introduced and at the same time the salt content of the ration was inadequate and probably the content of fat-soluble A low. Text-fig. 4 shows the curves of growth and Fig. 8 the condition of the animals at the time the experiment was discontinued. After 4 to 5 months growth had ceased, although at no time was it normal. The animals gradually passed into a poor condition, accompanied by lack of muscular coor-

dination, emaciation, and labored respiration. They were also unable to stand.

One was killed and the spinal cord was examined by Dr. Miller. Fig. 7 shows a section of the cord in the cervical region. Besides a considerable collection of fluid in the vertebral canal the motor cells were surrounded by fluid, compressed, the processes degenerated, and the general condition of the cord similar in kind, if not in degree, to those already described. In this cord there was a smaller number of motor cells in a degenerated condition than in some of the cords examined, but sufficient to connect directly the physical state of the animal with the condition found in the nervous tissue. It should be noted that in some cases the stainable material of the cell has collected on one side and in mass. The tigroid bodies are no longer discrete.

Experiment 5.—Since in all the experiments so far described wheat has either constituted a large percentage of the ration, or else its by-product, such as middlings, carrying the embryo, has made up a considerable fraction, we are led to the conclusion that the pathological condition of these animals resulted from toxicity inherent in the wheat. We believe that this is the correct explanation in these cases. However, evidence is accumulating from our experiments with swine that this pathological condition may probably be produced by rations free from any known toxicity and abundantly supplied with fat-soluble A and water-soluble B, but inadequate only in the quality and quantity of its mineral content. On a ration consisting of 45 parts of corn meal, 45 parts of oats, 10 parts of oil meal, and 5 parts of butter fat, the animal will, after a sufficient lapse of time, cease to grow and pass into a condition shown in Fig. 9 (Text-fig. 5 shows the curves of growth). No histological examination has yet been made of the cord of any of the animals of this group, and we withold final conclusions until more data are available; but the indications are rather positive toward the possibility of at least gross disturbances arising similar to those already described and involving the animal in external symptoms identical with those induced by the inherent toxicity of the wheat grain. In this ration the salt mixture is that common to the grains used. Unless later investigation should disclose toxicity in some of the products used, this would seem to be the logical explanation of our results.

Up to the present time we have found alfalfa meal or meat scraps among natural foodstuffs to be very successful supplements to a ration made up of corn, oats, middlings, and oil meal. There are undoubtedly many other materials that are either successfully in use or will be found capable of acting as supplements, but it is clear that if it is a single substance it must carry supplementing proteins, abundance of fat-soluble A, and especially a better salt mixture. We have no doubt that milk would readily come into this category if used in sufficient quantities to furnish an

adequate salt mixture. We have, however, records where skim milk was used in quantities sufficient to constitute as dry matter 1 to 5 per cent respectively of the dry matter of the ration. In these cases the grain ration was again made up of corn, oats, middlings, and oil meal. The protein level was approximately the same as in all the other experiments, 12 to 15 per cent. The results were a failure, as shown in the growth curves (Text-fig. 6) for the high intake of milk used. Failure was probably due to too low an intake of fat-soluble A and an inadequate supply of salts.

Where alfalfa constituted 20 to 25 per cent of the ration and middlings 20 per cent, the toxicity of the middlings was overcome, the inadequacy of the grains disappeared, and the animals remained sound and vigorous. Undoubtedly other roughages as the leaves or stems of plants would serve similar purposes. Curves of growth of these animals are shown in Text-fig. 7. This growth is somewhat below normal, possibly due to an absence of a highly efficient protein mixture.

As previously stated, it does not appear possible to supplement successfully the mixed grain ration here used with a single essential factor of growth. We have, however, in earlier experiments shown that the corn grain can be supplemented with salts alone with success in growth and maintenance, but here too the growth is somewhat below normal, due to poor proteins. With our mixed grain ration salts were used in the presence of middlings and a failure resulted. Butter fat has been imposed on the same ration and also on a mixture of corn, oats, and oil meal, with failure. It is probable, therefore, that our success with alfalfa rests on the introduction of a number of factors, such as more fat-soluble A and a better salt mixture. Whether the latter quality lies partly in its high calcium content remains for further study. These results with alfalfa make it clear how successful growth, even with omnivora, can be attained on a strictly vegetable diet,[12] and what an important part the leaf and stem portions of plants play in the life of herbivora.

With commercial meat scraps as supplements to a corn, middlings, oats, oil mixture as previously described, we have also had

[12] Hart and McCollum, *Proc. Am. Soc. Animal Production,* 1915; *J. Biol. Chem.*, 1916, xxiv, p. xxviii.

splendid success in obtaining normal growth and sustained vigor. This is a commercial product obtained from the packing establishments and carries considerable quantities of bone material. The calcium content of the material was 6.5 per cent. This product had been heated to 110°C., but the length of time could not be definitely given.

Curves of growth with both 1 per cent of meat scraps in the ration and with 5 per cent are shown in Text-figs. 8 and 9. These animals remained in splendid form and on 5 per cent have given birth to two litters of young. The reproduction, however, is not entirely satisfactory, a small percentage of the young being dead. Those that lived were vigorous and strong. On 1 per cent of meat scraps growth was splendid and maintenance perfect, but no successful reproduction has followed. The animals were physically active, conceived, but the young were carried from 1 to 2 weeks over time and all were born dead and hairless. On both diets carrying meat scraps the mothers have reached weights of 400 pounds or more. The factors introduced by this material were, again, better proteins, more fat-soluble A,[13] and an appreciable increase in calcium and phosphorus through the content of bone material in the meat scraps. It is indeed surprising to find as low an amount as 1 per cent of meat scraps supplementing these grains perfectly for growth, although apparently inadequate for successful reproduction. Our contention that alfalfa and meat scraps supplement the grains by virtue of introducing into the ration a better salt mixture, more fat-soluble A, and in the case of meat better proteins, is not wholly based on indirect inference, but on direct experimental inquiry. When a ration of corn, middlings, oats, and oil meal mixture has been supplemented with known salt additions and butter fat, thereby improving the mineral content of the ration and increasing the quantity of fat-soluble A, the curves of growth approach the normal and the animals appear at the present writing in normal condition. They have been on this ration for 100 days.

[13] Osborne and Mendel (*J. Biol. Chem.*, 1915, xx, 379) have shown the occurrence of fat-soluble A in beef fats.

SUMMARY.

Malnutrition, histologically characterized by nerve degeneration, may result from the absence of certain factors in the diet as in the case of beri-beri. A similar condition may likewise arise from the presence of toxic materials in apparently normal food products, and in the presence of all known factors essential for continued growth and well-being.

1. It is apparent from the results here recorded that with a large mass of wheat in the ration of swine toxicity will follow even in the presence of all the recognized factors for growth. Only in the presence of very liberal quantities of all these factors can the effect of the toxicity be overcome.

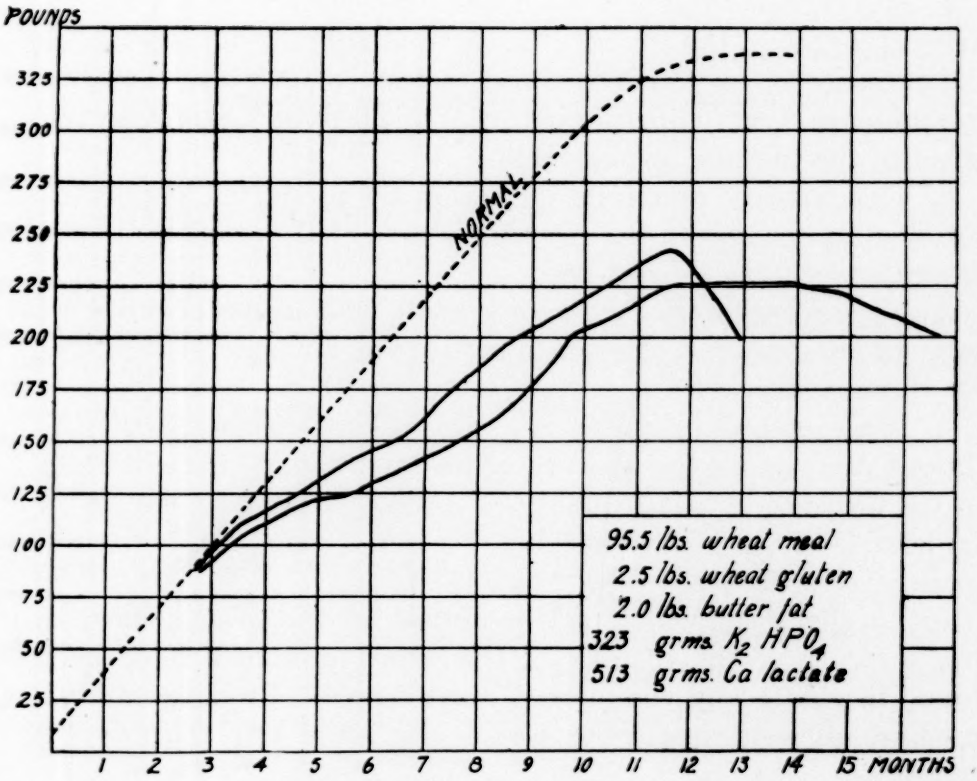
2. This toxicity manifests its action by producing important histological changes in the nervous system of the animal, not unlike those recorded for beri-beri.

3. No one important factor for growth, such as better proteins, salts, or fat-soluble A, appears able to act as a complete corrective for this toxicity.

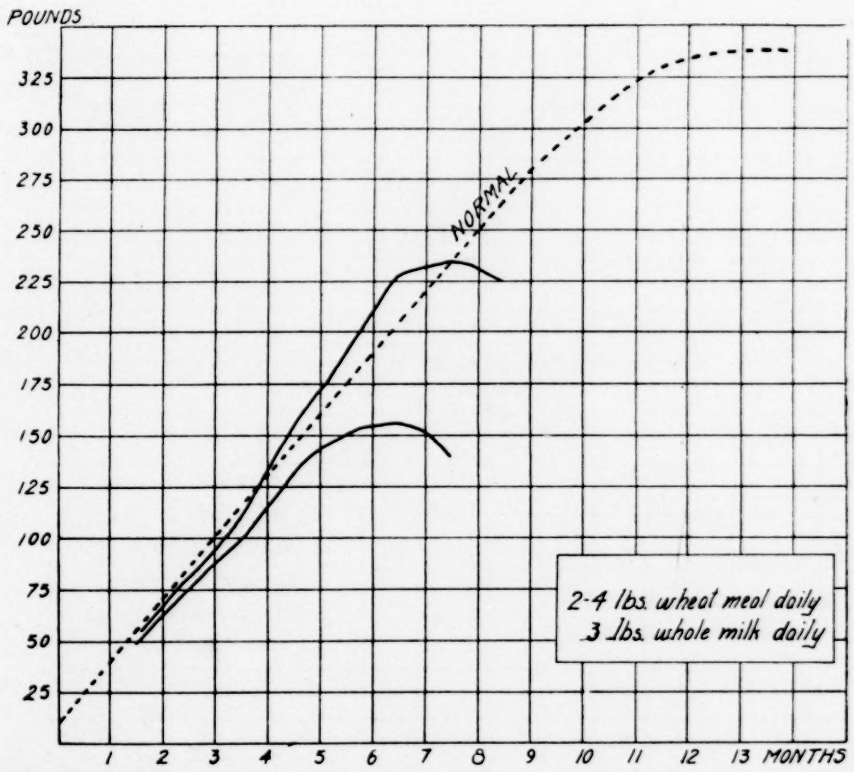
4. It also appears possible to produce similar pathological conditions in swine in the absence of all known toxic material and in the presence of a fair quality of protein, a plentiful supply of fat-soluble A, and water-soluble B, but a poor salt mixture; namely, that natural to the grains used.

5. Excellent supplementary materials to the grains, even in the presence of the toxicity of the wheat products, have been found in alfalfa and commercial meat scraps. Probably milk if used in sufficient quantities would also serve this purpose admirably.

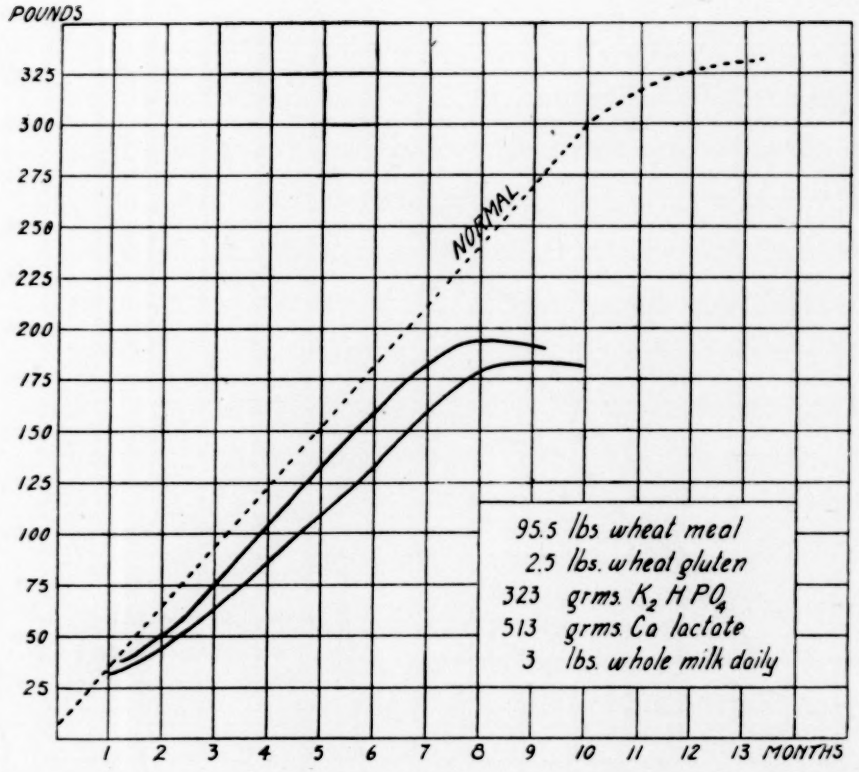
6. The factors introduced by alfalfa are undoubtedly an abundance of fat-soluble A and a better salt mixture. Its richness in calcium may be important and the relation of calcium to this problem will be studied further. With commercial meat scraps the factors for improvement are undoubtedly better proteins, more fat-soluble A, and a liberal supply of calcium phosphate resident in the bone material it carries.



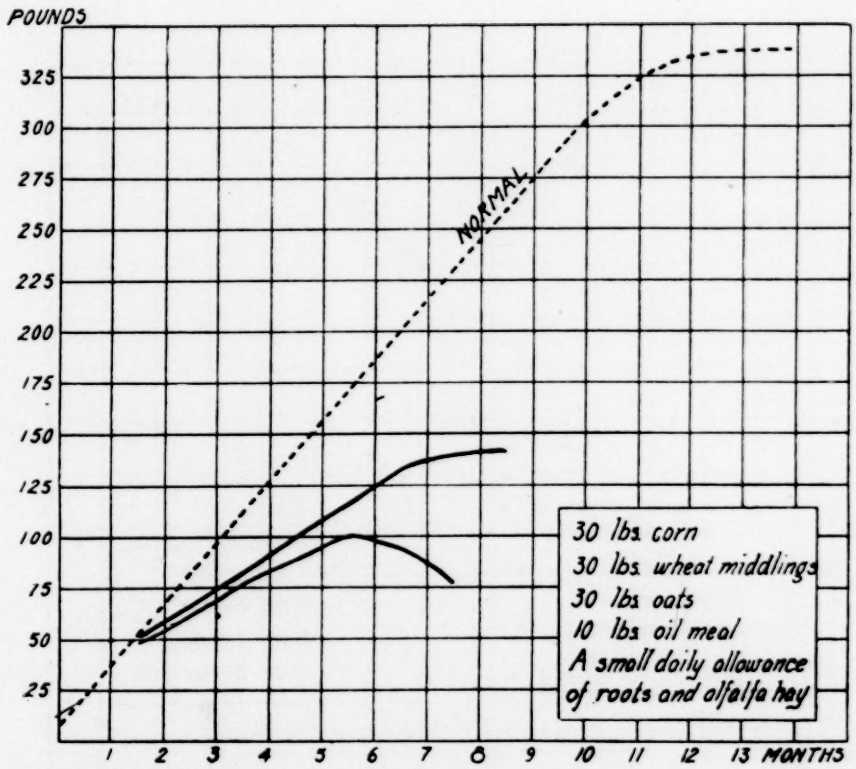
TEXT-FIG. 1. This shows the failure of swine to continue their growth on a ration of wheat, wheat gluten, butter fat, and salts.



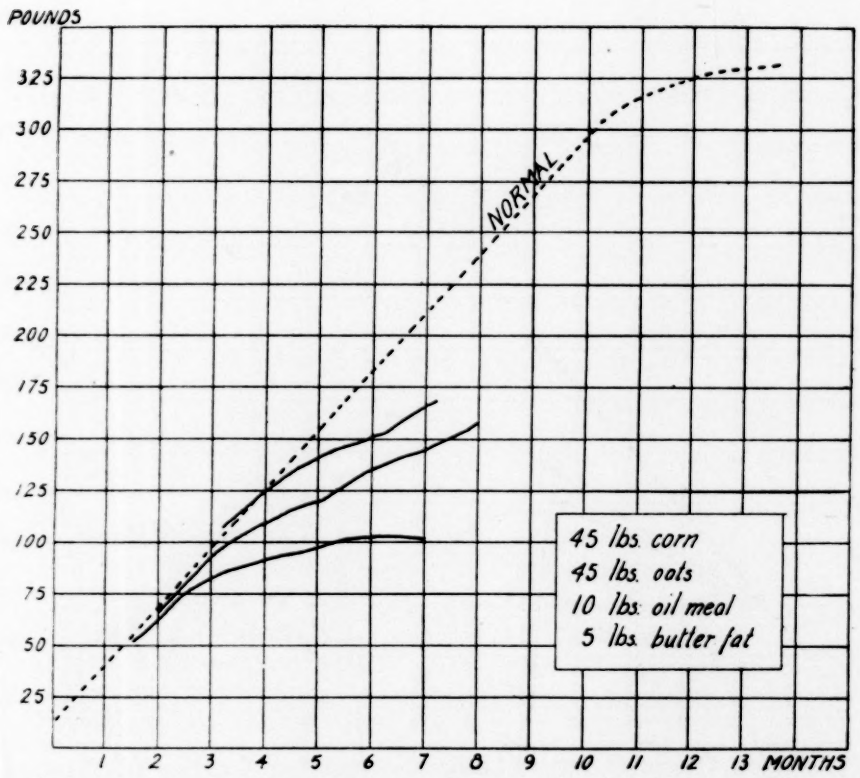
TEXT-FIG. 2. Growth curves of animals receiving wheat meal and 3 pounds of whole milk per individual daily. Growth was not sustained.



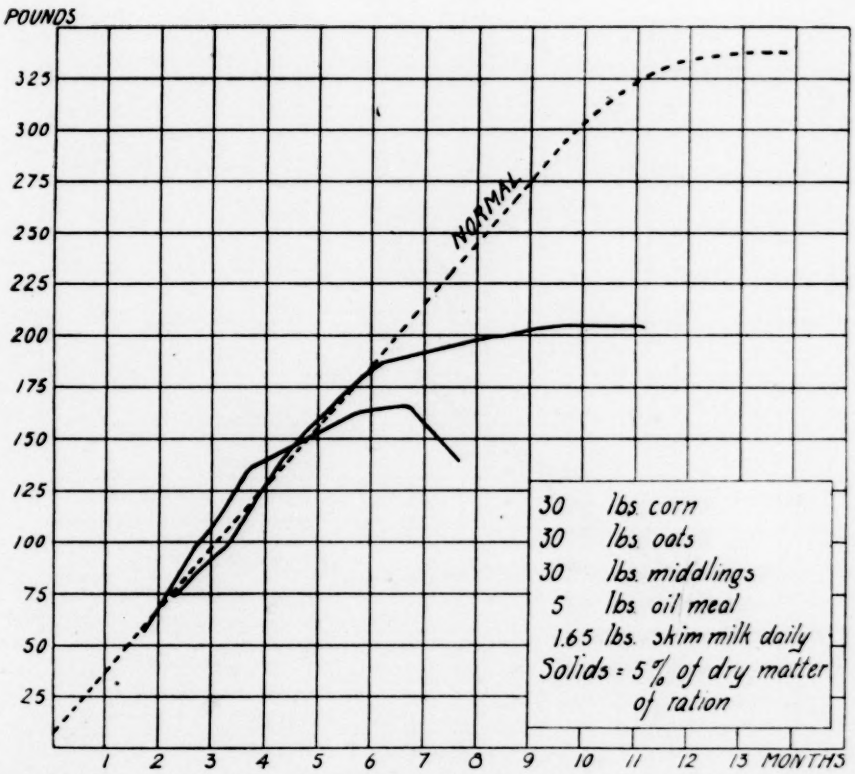
TEXT-FIG. 3. Growth curves of animals receiving wheat meal, wheat gluten, salts, and 3 pounds of whole milk daily per individual. Growth was not sustained.



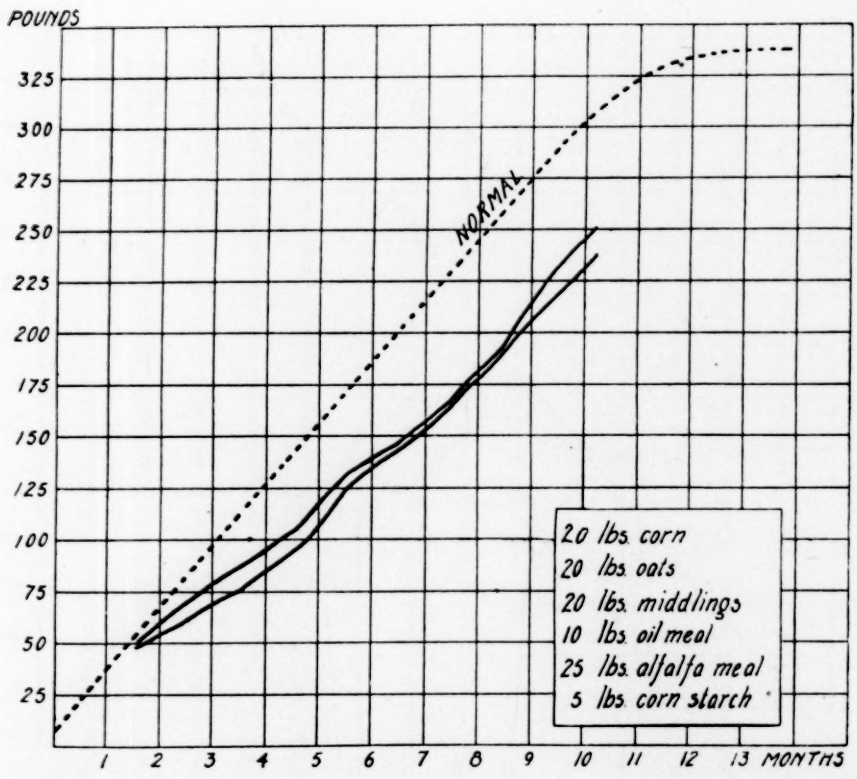
TEXT-FIG. 4. Growth curves of animals receiving a ration of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 10 parts of oil meal, and a quantity of alfalfa and roots constituting less than 5 per cent of the dry matter of the ration. Normal growth was not continuous and the animals passed into the condition shown in Fig. 8.



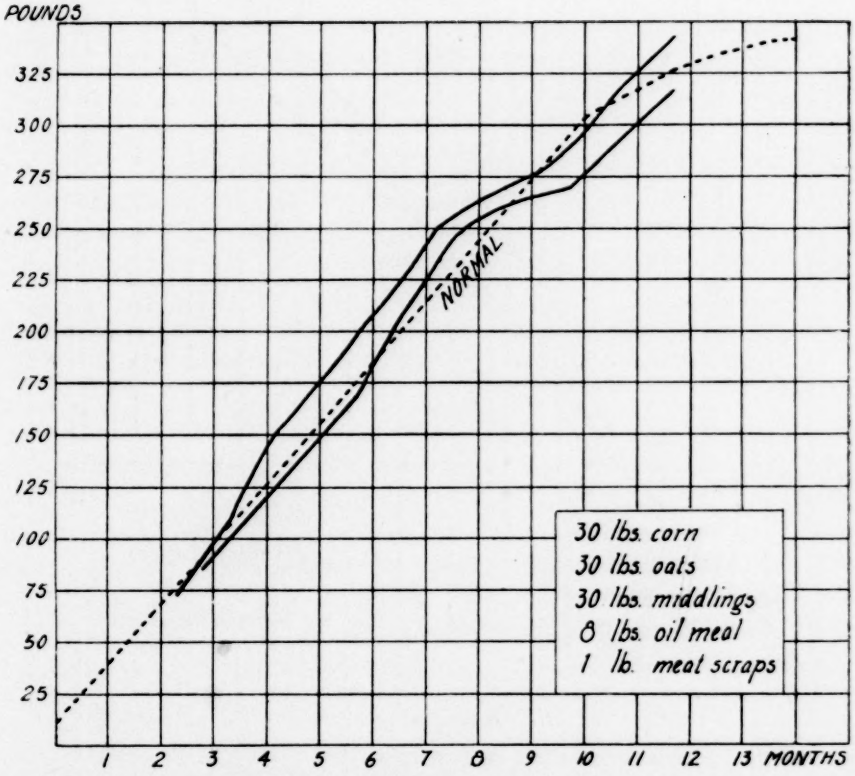
TEXT-FIG. 5. Growth curves of animals on a ration of 45 parts of corn meal, 45 parts of oats, 10 parts of oil meal, and 5 parts of butter fat. Growth was slow, below normal, and not sustained. These animals likewise passed into a poor condition. An insufficient salt mixture is probably responsible.



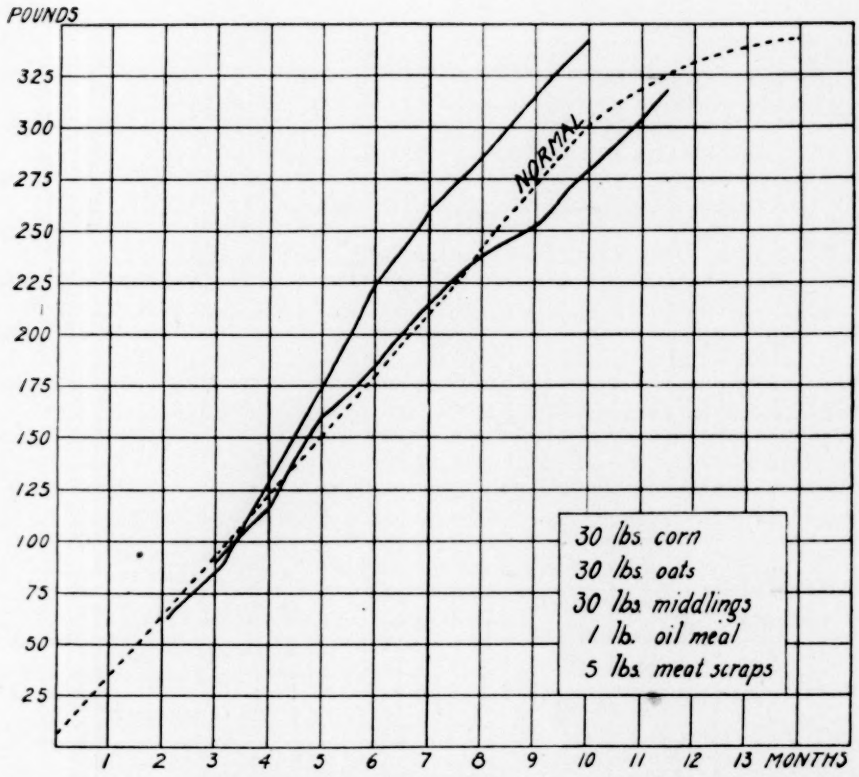
TEXT-FIG. 6. Curves of growth of swine receiving a grain mixture of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 5 parts of oil meal, and 1.65 pounds of skim milk daily. The mineral content of the ration and the quantity of fat-soluble A were probably insufficiently supplied to overcome the toxicity of the wheat middlings.



TEXT-FIG. 7. Curves of growth of swine on a ration made up of 20 parts of corn, 20 parts of oats, 20 parts of middlings, 10 parts of oil meal, 25 parts of alfalfa meal, and 5 parts of corn starch. The growth curve is a little below the normal, but physiological soundness was attained. The success secured here was probably due to a liberal supply of fat-soluble A and a better salt mixture, due to the introduction of the alfalfa meal.



TEXT-FIG. 8. Curves of growth of swine on a ration of 30 parts of corn, 30 parts of middlings, 30 parts of oats, 8 parts of oil meal, and 1 part of meat scraps. Normal curves of growth were obtained, but reproduction was not successful. The mothers conceived, developed the young, but they were born dead and hairless.



TEXT-FIG. 9. Curves of growth of swine on a ration of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 1 part of oil meal, and 5 parts of meat scraps. Growth was normal and reproduction of two litters has occurred. A small percentage of the young, however, were dead. A better protein mixture, more fat-soluble A, and an appreciable increase in the calcium content of the ration through the introduction of bone material with the meat scraps were probable factors in the successes exhibited in Text-figs. 8 and 9.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Animal receiving Ration 1, which consisted of wheat, wheat gluten, butter fat, and salts. Weight 206 pounds when photographed and losing weight rapidly.

FIG. 2. Cross section of a normal pig's spinal cord in the lumbar region. This animal was the same age as that shown in Fig. 1. Note that the motor cells show no constriction, that the nucleus and nucleolus are distinctly visible, and that the processes have not degenerated.

PLATE 2.

FIG. 3. Cross section of the spinal cord of the animal shown in Fig. 1 and receiving the wheat, butter fat, salt ration. Motor cells are constricted, processes degenerated, nucleus, nucleolus, and Nissl granules stained as a single bluish mass (black in photo). This condition is not unlike that described for polyneuritis in fowls.

FIG. 4. Animals receiving ration of whole wheat and 3 pounds of whole milk daily per individual. Prostrated when photographed.

PLATE 3.

FIG. 5. Cross section of the spinal cord of one of the animals shown in Fig. 4. This shows the condition of the motor cells in the thoracic region. There was marked edema and constriction of these cells.

FIG. 6. One of the animals receiving a ration of wheat meal, wheat gluten, salts, and 3 pounds of whole milk daily, the growth curves of which are shown in Text-fig. 3. The condition illustrated in this picture became typical soon after growth ceased.

PLATE 4.

FIG. 7. Cross section of the spinal cord in the cervical region of one of the animals shown in Fig. 8. The condition of a considerable proportion of the motor cells was similar to that shown in previous pathological cords where the diet carried a large percentage of wheat.

PLATE 5.

FIG. 8. Condition of animals after 4 to 5 months on a ration consisting of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 10 parts of oil meal, and a small quantity (less than 5 per cent) of alfalfa and roots.

FIG. 9. One of the individuals on the ration shown in Text-fig. 5. This consisted of 45 parts of corn, 45 parts of oats, 10 parts of oil meal, and 5 parts of butter fat. No wheat product was present and presumably no toxicity. The salt mixture was inadequate and this suggests the possibility of the poor salt mixture producing results similar to those produced by the toxic material of the wheat. Further work must fully decide this point.

FIG. 1.

FIG. 2.

(Hart, Miller, and McCollum: Deficiencies of Wheat and Grain Mixtures.)

FIG. 3.

FIG. 4.

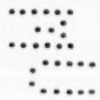
(Hart, Miller, and McCollum: Deficiencies of Wheat and Grain Mixtures.)

MU

FIG. 5.

FIG. 6.

(Hart, Miller, and McCollum: Deficiencies of Wheat and Grain Mixtures.)



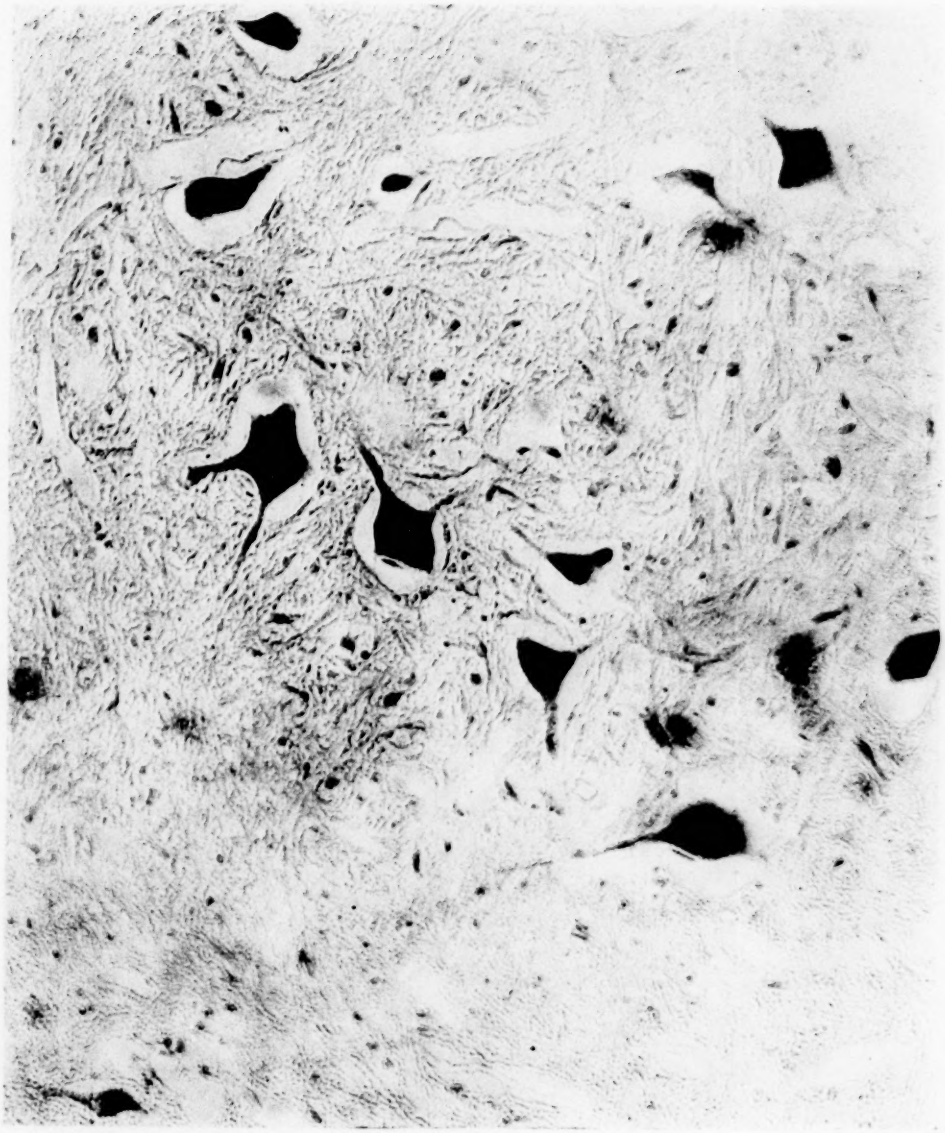


FIG. 7.

(Hart, Miller, and McCollum: Deficiencies of Wheat and Grain Mixtures.)

FIG. 8.

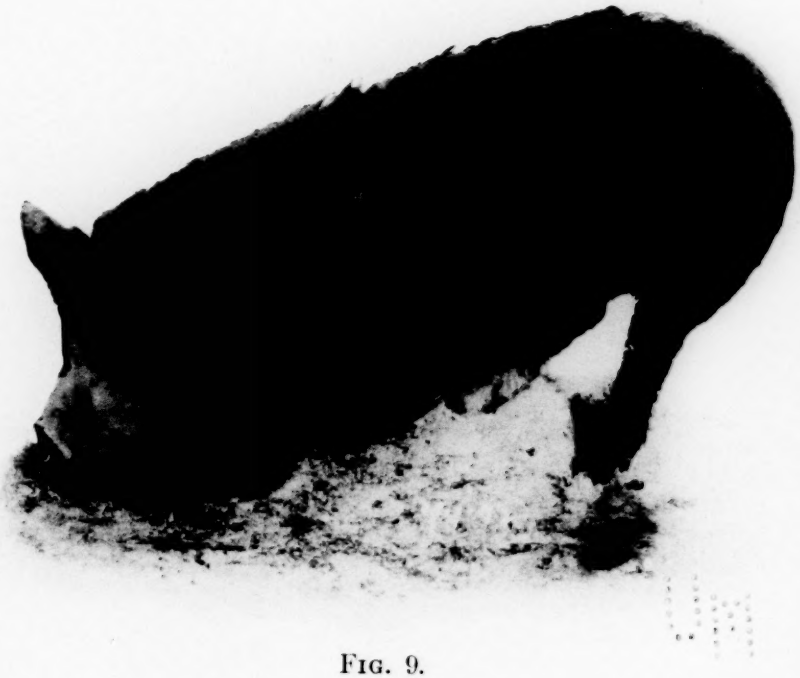


FIG. 9.

(Hart, Miller, and McCollum: Deficiencies of Wheat and Grain Mixtures.)

STUDIES OF AUTOLYSIS.

III. THE EFFECT OF REACTION ON LIVER AUTOLYSIS.

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In previous papers on the subject of autolysis[1] we have presented evidence which appears to indicate that the accelerating effect of acids and acid salts on the autolyzing liver is due to a change in the proteins from the resistant type which constitutes the bulk of the liver tissue to a form which will digest. While looking for activation of an enzyme as one of the factors governing the autolytic process we have thus far failed to find direct proof of any such activation. Indeed the failure of certain foreign proteins to digest at a more rapid rate in the presence of acids which greatly increase the rate and extent of liver-protein digestion, is direct evidence to the contrary.

In this paper we wish to present further data upon the determinative effect of *reaction* in liver autolysis with its bearing on the mechanism which controls the direction and extent of autolysis under physiological and pathological conditions. The experiments may be grouped as studies of the effect of acidity, neutrality, and alkalinity upon the digestion of liver proteins and of certain foreign proteins.

Methods.

In some of the experiments tannic acid was used as the protein precipitant, as described in previous papers. In others we have employed trichloroacetic acid as suggested by Greenwald,[2] titrat-

[1] Bradley, H. C., and Morse, M., *J. Biol. Chem.*, 1915, xxi, 209. Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113.

[2] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

ing the filtrates with the formol method or estimating the amino nitrogen by the Van Slyke micro apparatus. The trichloroacetic-acid-formol method has the great advantage of rapidity over the tannic acid technique. Inasmuch as only amino-acid carboxyl groups are titrated, the method necessarily gives a slightly different picture of autolysis than the soluble nitrogen method. In a general way, however, the two methods have been found to parallel each other closely, and the results of titration accord well with the results obtained by the Van Slyke method. The filtrates are always colored slightly yellow so that some practice is required in getting the end-points. 25 cc. of the digest are mixed with 50 cc. of trichloroacetic acid and made up to 100 cc. The mixtures are filtered after standing over night. 0.2 N NaOH is used for titration with phenolphthalein as indicator. The figures in the tables representing digestion are cc. of 0.2 N NaOH per 25 cc. filtrates where not otherwise designated.

I. The Effect of Acids and Acid Salts.

The liver proteins are very sensitive to acidity. In previous experiments the acidity of manganous salts, and of 0.01 N HCl, were shown to affect the rate and extent of autolysis markedly. We have here compared a number of other acids and salts.

1. CO_2.—The gas, thoroughly washed through $NaHCO_3$ and toluene water, was bubbled slowly through the digesting mixtures. Autolysis increased. This confirms the work of Yoshimoto with CO_2, boric, and salicylic acids.[3]

TABLE I.

Beef Liver.

	Days.					
	0	1	3	6	7	11
I. Control	0.40	1.80	2.40	2.70	2.60	2.60
II. " + CO_2	0.50	2.40	3.20	3.50	3.60	3.90

2. HCN.—The acid was prepared by several redistillations under diminished pressure from Merck's 5 per cent chemically pure

[3] Yoshimoto, S., *Z. physiol. Chem.*, 1908, lviii, 341.

acid. The original was contaminated with small amounts of the halogen acids and sulfuric acid and was found to give increased autolysis. The purified acid, whose strength was determined by silver precipitation, had no appreciable effect either upon the rate or the equilibrium of the digesting liver. HCN is known to be one of the weakest of the common acids. Made up with distilled water in strengths comparable to the concentrations used in the digests, it gave a $H^{\cdot}$ concentration of about $P_H = 6.0 \pm$, but was without measurable effect on the $H^{\cdot}$ concentration of a digest.[4]

TABLE II.

Horse Liver.

	Days.				Gain.
	0	2	5	10	
I. Control	0.40	0.95	1.15	1.20	0.80
II. " + HCN 0.02 N	0.40	1.05	1.30	1.30	0.90
III. " + " 0.01 N	0.40	1.00	1.25	1.20	0.80
IV. " + " 0.005 N	0.40	0.95	1.20	1.25	0.85

3. H_3PO_4 *and* KH_2PO_4.—Equivalent solutions of the salt and acid as measured by titration with NaOH give marked increases in autolysis. The acid shows the usual optimum concentration of about 0.04 N. The salt, on the other hand, even in 0.2 N strength, is less effective than the most dilute acidity tried—0.01 N. The $H^{\cdot}$ concentration in 0.01 N H_3PO_4 is higher than in 0.2 N KH_2PO_4.

[4] The hydrogen ion determinations cited in this paper were made with the Sörensen indicator method as applied by Rowntree and his coworkers (Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389). 5 cc. of the digest were dialyzed against 5 cc. of distilled water in steamed Jena glass for 3 minutes, using collodion bags. The phosphate solutions were corrected by titration but were not checked by the potentiometer, so that they are approximations rather than absolute figures.

TABLE III.

Beef Liver.

	Days.				Gain.
	0	2	6	12	
I. Control	0.80	1.25	1.90	2.55	1.75
II. " + KH_2PO_4 0.01 N	0.80	1.40	2.10	3.00	2.20
III. " + " 0.02 N	0.80	1.45	2.40	3.35	2.55
IV. " + " 0.04 N	0.80	1.70	2.60	3.60	2.80
V. " + " 0.1 N	0.80	1.90	2.80	3.90	3.10
VI. " + " 0.2 N	0.80	1.95	3.10	4.00	3.20
VII. " + H_3PO_4 0.01 N	0.80	2.00	3.20	4.05	3.25
VIII. " + " 0.02 N	0.80	2.30	3.60	4.50	3.70
IX. " + " 0.04 N	0.80	2.75	3.90	4.90	4.10
X. " + " 0.1 N	0.80	2.70	3.90	4.50	3.70
XI. " + " 0.2 N	0.80	1.20	1.20	1.35	0.55

4. Optimum Concentration of Various Acids.—The results tabulated below[5] are in confirmation and extension of the work of Arinkin.[6] Within rough limits the strong acids accelerate best between concentrations of 0.04 and 0.02 N when added to a 20 per cent liver digest. The less easily dissociated acids require higher concentrations for maximum effects. The evidence therefore points to the $H^{\cdot}$ concentration as being the factor of chief importance. As a general rule the optimum $H^{\cdot}$ concentration in such liver digests appears to be about $P_H = 6.00$, and filtrates or dialysates show a slightly acid reaction to Congo red.

TABLE IV.

Acid.	Optimum concentration.	Indicator reactions.
HCl	0.04–0.02 N	Congo red; slight.
H_2SO_4	0.02 N	" " "
H_3PO_4	0.04 N	" " "
KH_2PO_4	0.1 –0.2 N	" " "
Acetic	0.1 N	" " "
Lactic	0.04 N	" " "

[5] For many of the data in this experiment we are indebted to Miss Katherine Wright of this laboratory, in whose graduation thesis they appear.

[6] Arinkin, M., *Z. physiol. Chem.*, 1907, liii, 192.

II. The Effect of Neutral and Alkaline Reaction.

5. K_2HPO_4.—This salt is approximately neutral to litmus. In distilled water a 1 per cent solution has a H· concentration of $P_H = 6.85$. It is thus seen to be not far from the neutrality of water. Where present in an autolyzing mixture it will act as a buffer salt tending to maintain a constant H· level despite the normal formation of lactic acid in the tissue. Where small amounts are used the proportion of the acid phosphate formed will be sufficient to allow digestion to go on, and litmus acidity to develop within 24 to 48 hours. Where large amounts of the salt are added the inhibition of autolysis is marked. A concentration of 1 per cent is about as effective as that of 4 per cent.

TABLE V.

Beef Liver.

	Days.					Gain.
	0	1	2	5	10	
I. Control	0.70	1.40	2.20	2.80	3.10	2.40
II. " + K_2HPO_4 4.0 per cent	0.70	0.90	1.30	1.40	1.70	1.00
III. " + " 2.0 "	0.70	0.90	1.30	1.40	1.70	1.00
IV. " + " 0.8 "	0.70	1.00	1.30	1.60	1.80	1.10
V. " + " 0.2 "	0.70	1.20	1.60	2.00	2.45	1.75
I. Control	0.40		0.95	1.15	1.20	0.80
II. " + K_2HPO_4 2.0 per cent	0.40		0.70	0.80	0.75	0.35
III. " + " 0.8 "	0.40		0.80	0.85	0.80	0.40
IV. " + " 0.4 "	0.40		0.80	0.90	1.00	0.60
V. " + " 0.2 "	0.40		0.85	1.00	1.10	0.70
VI. " + " 0.08 "	0.40		0.80	1.20	1.20	0.80
VII. " + " 0.04 "	0.40		0.95	1.20	1.25	0.85

Noteworthy is the difference between the two beef livers used. One digested normally three times as fast and as far as the other. We have frequently observed such large individual differences between livers of the same species as well as between the livers of different species. It should be noted too that some digestion goes on in both cases even with a large excess of the buffer salt present. That the inhibition is not caused by a toxic effect of the salt molecule on the enzyme is clear from the fact that in-

creasing the concentration from 1 to 4 per cent does not increase the inhibition. The mechanism of inhibition seems rather to concern the suppression of developing acidity in the mixture.

6. $CaCO_3$.—The presence of this salt diminishes the speed of the reaction and lowers the point of equilibrium to a small extent. The insoluble carbonate will neutralize the developing lactic acid but will at the same time liberate an equivalent amount of CO_2. Since the digestion bottles remain corked, an acidity comparable to that of CO_2 will be the result. As has been shown, CO_2 bubbled into a normal digest where lactic acid is also developing unchecked will increase the digestion. In this case we find inhibition expressive of the substitution of the weaker acid in the digest.

TABLE VI.

Beef Liver. Tannic Acid Method.

	Days.					Gain.
	0	1	3	5	10	
I. Control	1.70	2.40	3.10	3.60	3.90	2.20
II. " + $CaCO_3$ 5 gm.	1.80	2.20	2.60	3.10	3.30	1.50

7. $MnCO_3$ *and* $CuCO_3$.—Manganous salts themselves exert no inhibitory effect upon the digestion of liver, but on the contrary may accelerate it. Copper salts, however, cause complete loss of activity. In these digests the insoluble manganous carbonate behaves much like the calcium salt, depressing the reaction slightly. Copper carbonate, however, allows no digestion to go on. Soluble copper salts are quickly formed in the mixture, as is indicated by the color of filtrates.

TABLE VII.

Pig Liver. Tannic Acid Method.

	Days.			Gain.
	0	3	6	
I. Control	1.40	5.10	5.50	4.10
II. " + $MnCO_3$ 5 gm.	2.60	4.30	4.70	2.10
III. " + $CuCO_3$ 5 "	1.40	1.40	1.30	0.00

8. Colloidal $Fe(OH)_3$.—The colloidal iron solution gave some acceleration in this case. The dialysate from it showed a H· concentration of $P_H = 6.1$, which is hardly sufficient to account for the acceleration. In any case the solution does not effectively neutralize developing acidity in any of the concentrations used.

TABLE VIII.

Pig Liver. Tannic Acid Method.

	Days.				Gain.
	0	2	4	10	
I. Control	0.90	2.82	3.70	4.40	3.50
II. " + 0.5 cc. solution	1.10	3.40	4.45	5.20	4.10
III. " + 1.0 " "	1.10	3.75	4.80	6.00	4.90
IV. " + 5.0 " "	1.15	3.60	4.90	4.90	3.75
V. " + 10.0 " "	0.80	3.80	3.60	4.00	3.20

9. ZnO.—The oxide was boiled with several volumes of water to remove any traces of soluble alkali. In the digests it prevents the development of acidity. The mixture reacts neutral to litmus throughout. Digestion is almost completely stopped. The inhibition is not due to a toxic action of the zinc salts on the enzyme, since we have shown previously that zinc sulfate accelerates autolysis. As with the neutral phosphate, a slight autolysis eventually shows itself, although within a 5 day period it is too small to measure.

TABLE IX.

Pig Liver. Tannic Acid Method.

	Days.					Gain.
	0	1	3	5	10	
I. Control	1.60	2.60	3.10	3.90	4.40	2.80
II. " + ZnO 5 gm	1.60	1.40	1.50	1.60	2.30	0.70

10. $NaHCO_3$.—Autolysis is inhibited by this salt in proportion to the concentration. The CO_2 liberated probably has some effect in counteracting the alkalinity of the salt in the lower concentrations.

TABLE X.

Beef Liver.

	Days.					Gain.
	0	1	3	5	10	
I. Control	0.50	2.30	2.35	3.10	3.40	2.90
II. " + $NaHCO_3$ 2 gm.	0.50	1.30	1.60	1.65	1.85	1.35
III. " + " 5 "	0.50	1.10	1.30	1.60	1.50	1.00

11. Strong Alkalies.—Na_2CO_3, NaOH, and MgO, when sufficient to make the reaction permanently alkaline to litmus, cause nearly complete stoppage of digestion. This confirms the results of Hedin and Rowland,[7] and von Drjewezki.[8] With decreasing amounts of hydroxide present, digestion proceeds proportionately better.

TABLE XI.

Beef Liver.

	Days.					Gain.
	0	1	2	5	10	
I. Control	0.70	1.40	2.10	2.80	3.10	2.40
II. " + 50 cc. 0.2 N NaOH	0.70	0.80	0.80	0.90	1.00	0.30
III. " + 40 " 0.2 N "	0.70	—	0.90	1.00	1.30	0.60
IV. " + 30 " 0.2 N "	0.70	—	1.10	1.00	1.25	0.55
V. " + 20 " 0.2 N "	0.70	—	1.10	1.10	1.40	0.70
VI. " + 10 " 0.2 N "	0.70	1.10	1.30	1.60	1.70	1.00
VII. " + 5 " 0.2 N "	0.70	—	1.50	1.90	2.35	1.65

In this experiment it is evident that the hydrolysis is not due to the alkali itself since the highest concentrations give the smallest figures. Where 20 cc. or less of the hydroxide are present the reaction becomes neutral or acid to litmus within 2 days. The digestion in these samples is referable to the excess of acid developed in the mixtures.

To sum up this portion of the data, it seems clear that any compound which tends to keep a liver digest close to litmus neutrality also tends to inhibit the digestion, reducing it to a minimal figure or even to zero in some cases. In the presence of al-

[7] Hedin, S. G., and Rowland, S., *Z. physiol. Chem.*, 1901, xxxii, 341, 531.
[8] von Drjewezki, A., *Biochem. Z.*, 1906, i, 229.

kalinity sufficient to be indicated by phenolphthalein, digestion is stopped and it can easily be shown that the enzyme is destroyed. This inhibitory action of neutral or alkaline reaction may be due to the following mechanisms: (1) The enzymes present may require a certain $H^{\cdot}$ concentration before they become active. (2) The salts added may cause a change in the normally available proteins rendering them less digestible. (3) The bulk of liver proteins may be unavailable during normal conditions within the cell and require the developing acidity of the dead or dying tissue to convert them into digestible form. (4) Combinations of these factors may constitute the actual mechanism.

To elucidate this point further we have undertaken to determine the effect of the reaction of the mixture upon the hydrolysis of foreign proteins. It is evident that the activity of the enzymes can only be determined by measurements of hydrolytic change. Where the mass of substratum derived from the liver is so sensitive to small changes of reaction we must depend upon measurements of the rate of digestion of foreign proteins which are found to be less sensitive, in order to get direct evidence of the degree of activity of the enzymes. In a previous paper[9] casein and peptone were found to be digestible by the normal liver enzymes. Adding acid to the liver had no effect upon the rate of the digestion of these foreign proteins. We concluded that the addition of acid to the normal liver digests did not increase the activity of the proteases present *as measured by the rate of digestion of casein and peptone.* This fact does not rule out the possibility that liver proteases become active at some relatively low $H^{\cdot}$ level, such as we have at the beginning of autolysis, or soon after the death of the tissue.

III. Digestion of Foreign Proteins.[10]

12. Casein and Peptone with $CaCO_3$.—The peptone used was prepared from Witte's peptone by solution in water and repeated precipitation with alcohol. The solution was neutral to litmus.

[9] Bradley, *J. Biol. Chem.*, 1915, xxii, 113.

[10] We assume here that the liver enzymes which we have found able to digest peptone, casein, gelatin, and certain other proteins, are also the enzymes which digest the liver proteins. There is no reason for supposing a specific enzyme for each protein.

Casein was dissolved in NaOH and neutralized to litmus. The H· concentration of this solution was found to be $P_H = 6.47$, or about that of normal liver digests after 1 or 2 days' autolysis.

TABLE XII.

Pig Liver. Tannic Acid Method.

	Days.					Gain.	Digestion of foreign protein.
	0	1	3	5	10		
I. Control	1.70	2.40	3.10	3.60	3.90	2.20	—
II. " + $CaCO_3$ 5 gm.	1.80	2.20	2.60	3.10	3.30	1.50	—
III. " + casein	2.00	7.10	8.60	9.00	10.10	8.10	5.90
IV. " + {casein, $CaCO_3$ 5 gm.	2.00	6.20	7.60	8.00	8.80	6.80	5.30
V. " + peptone	3.10	6.40	7.50	7.60	8.60	5.50	3.30
VI. " + {peptone, $CaCO_3$	3.00	6.30	7.50	8.30	9.00	6.00	4.50

There appear to be a very slight inhibition of casein digestion and an increased digestion of peptone. If the figures are analyzed to determine the rate of change, it will be found that casein digests more slowly during the first 24 hours in the presence of the carbonate, while the peptone digests more rapidly. Under the same conditions the liver digests at about half its normal rate. The experiment shows a lack of uniformity in the effect produced upon the three digestible proteins—liver, casein, and peptone. This lack of uniformity will be found throughout the experiments which follow and constitutes a strong argument against activation of the enzyme as a determinative influence in autolysis.

13. Peptone with ZnO *and Alkalies.*—In this experiment liver digestion is reduced to about 25 per cent of its normal figure in the presence of ZnO; the digestion of peptone to about 60 per cent. In the presence of MgO liver is reduced to 20 per cent of the normal, while peptone is reduced to about 45 per cent. Where there was insufficient Na_2CO_3 to maintain litmus neutrality throughout the autolysis, liver digestion was reduced to 50 per cent of the normal while peptone digested to 90 per cent of the normal.

TABLE XIII.

Beef Liver. Tannic Acid Method.

	Days.					Gain.	Digestion of foreign protein.
	0	1	2	5	9		
I. Control	1.60	2.60	3.10	3.90	4.40	2.80	—
II. " + ZnO	1.60	1.40	1.50	1.60	2.30	0.70	—
III. " + MgO	1.60	1.70	1.40	1.50	2.20	0.60	—
IV. " + peptone	2.80	6.40	6.70	7.90	9.00	6.20	3.40
V. " + {peptone, ZnO}	2.80	4.00	4.20	4.60	5.60	2.80	2.10
VI. " + {peptone, MgO}	2.70	4.10	4.00	4.00	4.70	2.00	1.40
VII. " + Na_2CO_3	1.60	1.90	2.00	2.60	3.00	1.40	—
VIII. " + {peptone, Na_2CO_3}	3.00	6.00	5.90	6.10	7.40	4.40	3.00

Again if we determine the rate it is found that in the first 24 hours there is no digestion of liver with ZnO and MgO. Under the same conditions peptone digests at 50 per cent of its normal rate. With the carbonate present the rate of liver digestion is about 30 per cent of the normal, while the peptone digests at full normal rate.

14. Casein with ZnO *and* MgO.—In the presence of the oxides, liver shows no digestion in a 5 day period. With ZnO casein is reduced to 30 per cent of its normal rate in the first 24 hours, with MgO to 17 per cent. The rate of liver digestion is zero. In order to eliminate the error which might result from hydroly-

TABLE XIV.

Beef Liver. Tannic Acid Method.

	Days.				Gain.	Digestion of foreign protein.
	0	1	3	5		
I. Control	2.20	4.70	5.55	5.60	3.40	—
II. " + ZnO	2.20	2.20	2.00	2.20	0.00	—
III. " + MgO	2.20	2.40	2.40	2.20	0.00	—
IV. " + casein	2.30	8.80	9.50	9.80	7.50	4.10
V. " + {casein, ZnO}	2.30	3.50	3.90	4.30	2.00	2.00
VI. " + {casein, MgO}	2.30	3.00	3.15	3.10	0.90	0.90

sis of the foreign proteins by the alkali alone we have digested casein and peptone with ZnO and MgO at the same temperature and for the same length of time. No hydrolysis occurs.

15. Casein and NaOH.—Progressive additions of NaOH produce the graded inhibition of liver autolysis reported in Table XI. The effect of the alkali upon casein digestion is quite otherwise. Even in the higher concentrations of the hydroxide casein digests better than in the normal control.

16. Peptone and NaOH.—In a single experiment sufficient NaOH was added to reduce liver autolysis to 60 per cent of the normal. Peptone digested slightly better than in the normal.

17. Casein and K_2HPO_4.—Adding progressive amounts of the salt, as in Table V, with and without casein, we find parallel inhibition of both liver and casein digestion.

18. Casein and $NaHCO_3$.—With varying amounts of the salt, sufficient to reduce liver digestion to less than 50 per cent of the normal amount, casein digested better than in the normal, except in the highest salt concentration of 2 per cent.

19. Gelatin and $NaHCO_3$.—The digestion of gelatin which occurs in the normal liver mixture is inhibited by the addition of the salt, but to a less extent.

TABLE XV.

Beef Liver.

	Days.					Gain.	Digestion of gelatin.
	0	1	3	5	10		
I. Control	0.50	2.30	2.35	3.10	3.40	2.90	—
II. " + gelatin	0.80	3.30	5.10	5.25	5.75	4.95	2.05
III. " + $NaHCO_3$ 2 gm.	0.50	1.30	1.60	1.65	1.85	1.35	—
IV. " + gelatin, $NaHCO_3$ 2 "	0.80	2.30	3.25	—	3.80	3.00	1.65
V. " + NaHCO 5 "	0.50	1.10	1.30	1.60	1.50	1.00	—
VI. " + gelatin, $NaHCO_3$ 5 "	0.80	2.10	2.75	2.95	3.25	2.45	1.45

It is of interest to note that in this liver gelatin digests at the identical rate in the three mixtures for the first 24 hours. At the beginning of autolysis, therefore, the rate was the same

whether the salt was present or not. The following analyses of the data will illustrate this point:

Control gains 1.80 cc. during the first 24 hours.
Control + gelatin gains 2.50 cc.
Gelatin alone (difference) 0.70 cc.

Salt mixture (2 gm.) gains 0.80 cc.
Salt mixture + gelatin gains 1.50 cc.
Gelatin alone 0.70 cc.

Salt mixture (5 gm.) gains 0.60 cc.
Salt mixture + gelatin gains 1.30 cc.
Gelatin alone 0.70 cc.

This point we wish to emphasize because it clearly shows that the normal and alkaline liver mixtures are equally active as judged by the speed of gelatin digestion during the early period of the reaction, when differences in speed would be readily recognized. Since the amount of gelatin added was the same in each case, equal rates of digestion indicate equal amounts of enzyme, or equal activity of enzyme. As far as the experiment goes it tends to negate a change in activity of the enzyme which digests gelatin, even in the presence of considerable bicarbonate. Since gelatin is a rather non-specific type of protein, digesting in most protease solutions, it seems fair to conclude that the same enzyme which digests the gelatin also digests the liver proteins. If this assumption is correct, there is no indication of loss of enzyme activity even at the low $H^{\cdot}$ level of this experiment.

20. NaOH *and Hemoglobin.*—Dog hemoglobin was found to digest slowly in an autolyzing liver. The addition of NaOH (25 cc. 0.2 N solution in 250 cc. liver mixture) inhibited the digestion of liver and of hemoglobin equally. A powdered fibrin preparation was found not to digest in either case.

In summarizing the effect of varying reaction of the liver mixtures upon the digestion of foreign proteins, it is clear that no parallelism has been found. Digestion of liver may be almost completely stopped by a salt which even accelerates the digestion of casein by the liver. Peptone may digest at its normal rate in a liver which is reduced to half its normal speed of autolysis

by the reaction. Gelatin may digest at normal speed in a mixture which reduces the speed of liver digestion to a third of the normal. Hemoglobin may show equal inhibition with liver. Acids which cause liver to digest much more rapidly and completely do not alter the rate of digestion of casein and peptone. Such variations do not indicate such a common factor as the activity of an enzyme. They rather indicate that the various proteins used, including the liver proteins, may or may not be altered by the presence of acids or salts or alkalies in the same direction or to the same extent.

From the spleen Hedin[11] isolated two enzymes, α and β proteases, active in alkaline and acid media respectively. If we assume that the liver contains these enzymes in significant amounts also, then it is apparent that the α protease is of no moment in the autolysis, since under conditions of neutrality or alkalinity there is practically nothing to digest. Nor does the possible activation of the β protease appear at all determinative in autolysis since the enzyme appears to be fully active at a $H^{\cdot}$ level at which there is little or no available protein substratum. The effect of reaction upon liver autolysis may be broadly stated as follows:

1. The progressive addition of alkali, or of salts like $NaHCO_3$ and K_2HPO_4, reduces the normal rate and extent of liver autolysis in rough proportionality to the amount added, until complete or nearly complete inhibition results.

2. The progressive addition of acids and acid salts leads to an increase in the rate and extent of liver autolysis in rough proportionality to the amount added.

3. In progressive alterations of reaction from faint alkalinity to litmus to an acidity which may be just recognized by Congo red, and in these experiments amounts to a 0.04 N solution, we may pass from zero digestion (or a low minimum figure) to 90 per cent of the total protein of the tissue, and this would approximate 100 per cent digestion if the connective tissue fraction could be eliminated.

4. Such progressive alterations of the speed of digestion and the point of equilibrium do not appear to be correlated with any cor-

[11] Hedin, S. G., *J. Physiol.*, 1903–04, xxx, 155.

responding change in enzyme activity, *as measured by the digestion of foreign proteins.*

5. Such progressive alterations of speed and equilibrium are of just the type produced by progressive changes in the mass of substratum in the digest.

We therefore conclude that the effect of reaction in a liver digest is to alter the liver proteins, increasing or decreasing the mass of available substratum as the reaction becomes more or less acid. Soon after death the normal liver is found to give a dialysate with a $H^{\cdot}$ concentration averaging about $P_H = 7.00$. At the moment of death it is probably much lower and approximates the reaction of blood, $P_H = 7.40–7.80$. At these levels little or no autolysis takes place. Unless prevented, the development of acid is very rapid and produces a rapid rise in the $H^{\cdot}$, as Morse has recently shown.[12] Such an increase will determine the amount of actual substratum that becomes available in the normal liver for autolytic proteolysis. We feel confident in asserting that the variations which one finds in the normal autolysis of various livers will be found to correspond to the amount of acid produced. For example, the liver of a rabbit is as a rule slow to autolyze and reaches a low point of equilibrium normally. The liver of a dog, on the other hand, digests more rapidly and to a higher amino-acid level. The individual differences can be made to disappear, however, by artificially controlling the acidity. Thus rabbit liver may be made to digest like dog liver by adding the proper amount of acid, and dog liver can be reduced in its digestion to that of rabbit liver by the addition of the proper amount of K_2HPO_4. The rabbit liver probably produces on the average less acid than does that of the dog. The extent of individual variations in the same species may be quite large. In Fig. 1 are shown the limiting variations and the mean of eight dog livers —all apparently normal. Seven of the eight curves fell within the limits of 1 and 2. The curve of the eighth dog liver is No. 3 and is evidently abnormal, though the dog was apparently perfectly normal and the liver, to outward appearances, was so.

[12] Morse, M., *J. Biol. Chem.*, 1916, xxiv, 163.

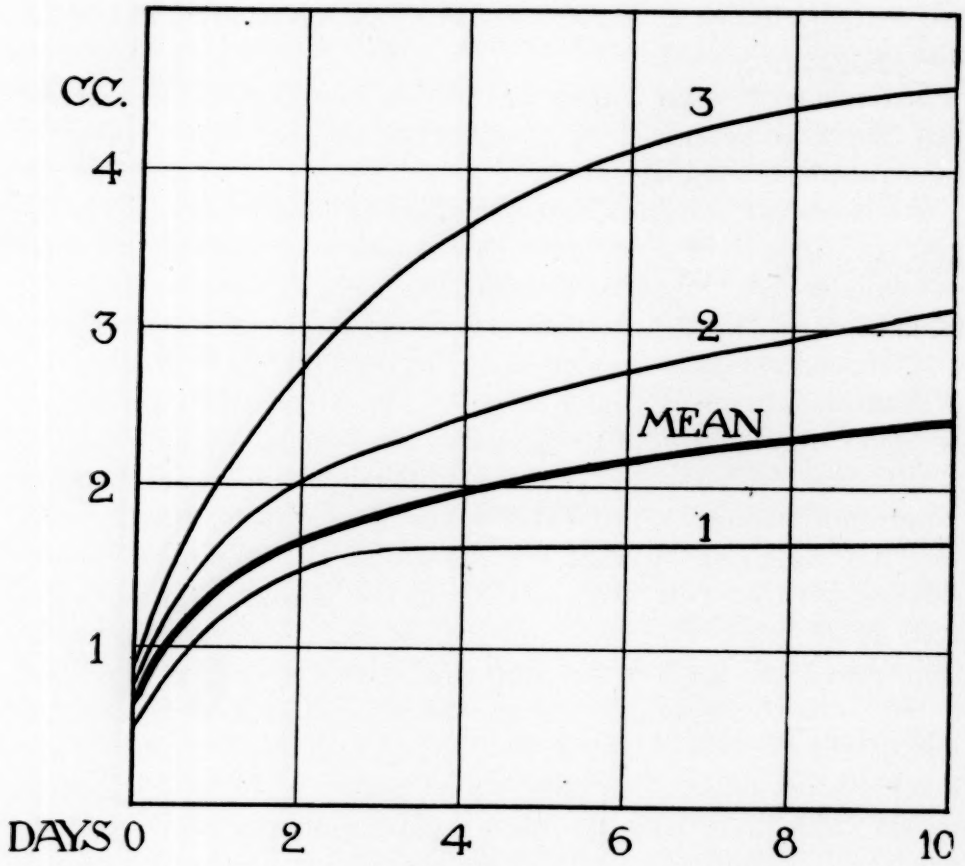

FIG. 1.

The shift from non-available proteins to available is evidently a reversible reaction depending for its equilibrium upon the H· concentration of the cell at the given moment. The non-available protein fraction may be a protein salt, or a polymerized protein aggregate of large size. It is well known that many polymerizations are facilitated by an alkaline reaction. The available protein fraction may be a dissociated protein salt, or a more highly dispersed colloid. The charge on the colloid particles may determine availability. Without wishing to commit ourselves as to just what molecular changes are involved in the shift from one type to the other with a change in the H· concentration, we wish to illustrate by the following diagram what we conceive to be the relationships existing in a liver cell.

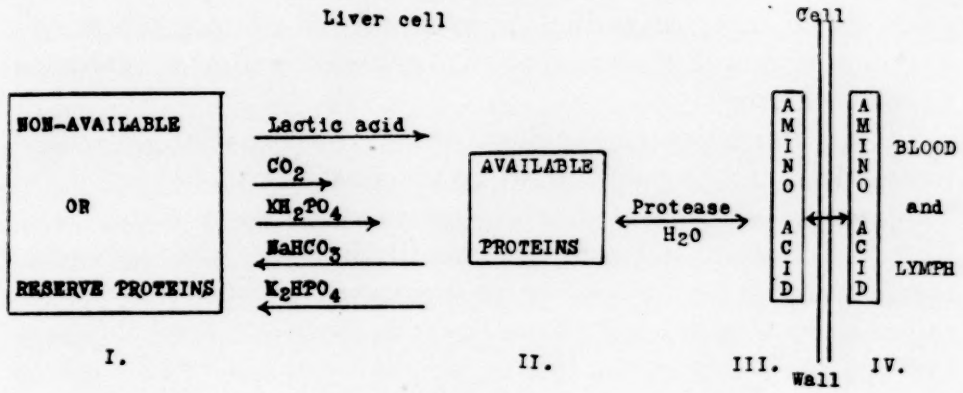

FIG. 2.

In this diagram we assume the reversibility of protein hydrolysis facilitated under proper conditions by the proteases present. While perhaps never satisfactorily proven, such evidence as we have points to the validity of this assumption.

It is evident that with no change in the $H^{\cdot}$ level in the cell, no change in the relation of I to II would occur. With increased acidity—be it only a sudden increase in the CO_2 content of the cell—some of I is converted into II. If II is in equilibrium with its hydrolytic products any increase in the mass of II would upset the balance between II and III, digestion would begin, the amino-acid content of the cell would rise with diffusion out into blood and lymph until equilibrium between II: III: IV was again established. Mass relationships govern the equation II: III, but the salts of the blood and cell, or the $H^{\cdot}$ concentration, govern the equation I: II.

In the following paragraphs we wish to apply this working hypothesis tentatively in the explanation of some of the more common phenomena of autolysis in the living organism. What follows, therefore, is not presented as fact, but as observations which appear to fall in line with the data and views presented in this and previous papers. Each point will require individual testing and will constitute the basis for subsequent contributions from this laboratory.

In acidosis there is a shift in the salt balance of the blood. By diffusion this shift would be communicated to the cell. A corresponding shift in the substratum would result, with an upsetting of the equilibrium II: III in favor of hydrolysis. Amino-

acids would increase within the cell and diffuse into blood and lymph. It is well known that nitrogen elimination is increased in severe acidosis.

During starvation the acidosis which appears after a variable fore-period, and is believed to be associated with imperfect fat combustion, is correlated with increased nitrogen elimination. The mobilization of tissue proteins in the form of amino-acids may indeed depend upon, or be facilitated by this shift in the salt balance of blood and tissues toward a higher $H^{\cdot}$ level. Schryver[13] points out that the loss in weight by the liver and spleen during starvation is unusually large. Both of these organs are very sensitive to a change in the $H^{\cdot}$ concentration, as shown by autolysis.

In local areas diminished blood supply leads to partial asphyxia, poor removal and neutralization of CO_2 and acid products of incomplete combustion. In such local areas we find atrophy, shrinkage, or vacuolation of the cells, changes in the staining reactions—all expressive of autolysis and associated with the shift in the available proteins of the cell caused by shift in acidity. Such atrophy may be induced experimentally by ligation of arteries, by embolism, or by thrombosis.

The presence in the cell of any substance preventing the normal oxidations should lead to the formation of intermediate acid products. Phosphorus, a powerful reducing agent and itself oxidizing under certain conditions to a strong acid, is well known for its disintegrating effect upon the liver.[14] We believe that part of the effectiveness of phosphorus in producing rapid degeneration of the liver is due to the production of small amounts of phosphoric acid within the cells themselves, as well as to the prevention of normal oxidations of the cell substances so that the production of organic acids results. At least it is suggestive that phosphorus has little accelerating effect on a liver autolysis unless oxygen be admitted to the digest. We expect to present further data on this point later.

Graham[15] believes that chloroform poisoning results in the formation of HCl within the liver cells with a rise in the $H^{\cdot}$ con-

[13] Schryver, S. B., *Biochem. J.*, 1906, i, 123.
[14] Jacoby, M., *Z. physiol. Chem.*, 1900, xxx, 174.
[15] Graham, E. A., *J. Exp. Med.*, 1915, xxii, 48.

tent as shown by indicators. Such a rise would increase the available substratum and cause autolysis. Acute atrophy of the liver is characteristic of chloroform poisoning.

Pressure on an organ leads to cutting down the blood and lymph flow within the compressed area. Partial asphyxia in such an area leading to the accumulation of acid products would thus increase autolysis and lead to the well known pressure atrophies. Pressure and alteration of the blood supply probably cause the atrophy of the mammary gland after lactation, by the same mechanism.

Alterations in the blood supply of the uterus after parturition probably cause a shift toward acidity in the cells and thus a change in the proteins resulting in the rapid atrophy of that organ. Although we have not as yet studied the effect of increased acidity on the autolysis of the uterus it will in all probability be found to react as does the liver, though to a less marked degree.

In the rapidly growing tumors, especially carcinomata, we find the mass of cells outgrowing the blood supply, or by compression changing it to certain areas of the tumor. Such rapidly growing tissues are especially liable to necrosis and extensive autolysis. The onset and the extent of this autolysis is probably determined by partial asphyxia of masses of cells, accumulation of acid products, the shift in the proteins, and their consequent digestion.

Hypertrophy itself seems, as a rule, to be conditioned by an increased blood supply. Such an increase would insure complete oxidation, removal of CO_2, neutralization of organic acids if any formed, and a constantly renewed supply of the phosphate mixture of the blood. The salt balance of the cells would therefore tend to remain approximately that of the blood, with a $H^{\cdot}$ concentration[4] of $P_H = 7.4$–7.8. Such a condition would reduce autolysis to a minimum by converting all the available proteins into the reserve type. If more of the available type were synthesized more of the reserve fraction would accumulate. The tendency would therefore be to increase the cell proteins to the maximum, and the result would be a simple hypertrophy. In certain tissues this might be accompanied by cell division and a numerical hypertrophy as well. One of the important factors limiting this process of growth or hypertrophy will be the relation of the tissue to its blood supply—governed automatically by

the $H^{\cdot}$ level maintained in the tissue and its effect on the active masses of substratum in the cells. A point is eventually reached where further additions to the tissue mass must constitute an excess of demand over supply of oxygen, or mechanically prevent adequate movement of lymph and blood. A condition of equilibrium is the result, such that further tendency to growth is counterbalanced by a rising of the $H^{\cdot}$ level in some of the cells least favorably located in relation to the blood, with consequent tendency toward autolysis, and shrinkage.

Hypertrophy, atrophy, and maintenance of tissue mass are, we believe, governed by some such fundamental mechanism as we have described. Innumerable complicating factors must intervene to modify the final condition of a tissue, its rate and direction of change; but it seems likely that in all such tissues as are sensitive to increased acidity and respond by increased autolysis, the determining mechanism is the one described above for the liver.

SUMMARY.

1. Progressive changes in the reaction of autolyzing liver tissue, from an alkalinity expressed by $P_H = 7.4$ to the optimum acidity of $P_H = 6.0$ (measured in the dialysate), cause proportionate changes in the rate and extent of autolysis from nearly zero to 90 per cent digestion.

2. Under these same conditions of varied reaction certain foreign proteins digest without indicating any change which could be interpreted as an activation of an enzyme.

3. The effect of such changes in the reaction of the tissue is to alter the mass of digestible protein present in the liver from nearly zero, when alkaline, to nearly complete digestibility at an acidity of $P_H = 6.0$.

4. A tentative working hypothesis based upon these facts is presented and applied to a number of well known conditions which involve autolysis or the reverse process of hypertrophy in the living organism.

5. Atrophy, involution, necrosis, hypertrophy, and tissue equilibrium in such tissues as respond to increased acidity by increased autolysis, appear to depend upon the mechanism described as of fundamental importance in determining the direction, rate, and extent of tissue change.

STUDIES IN BENCE-JONES PROTEINURIA.

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The subject of these studies is an intelligent patient under the care of Dr. C. H. Schoff, of Media, Pa., who supplied the following summary of the history.[1]

J. E. L. Age 52. White. Male. Childhood unimportant. When 26 he began to lose weight and after a short period of ill health had a hemorrhage from the lungs. After 2 years in the West he entirely regained weight and health. 4 years ago he commenced having attacks of bronchitis accompanied by severe shooting and aching pains in the back and legs. The last severe attack of these pains commenced in February, 1915, and continued for several months. In April of that year Bence-Jones protein was first discovered in the urine. There were at times cough and expectoration, but never tubercle bacilli in the sputum. A few of the latter were at one period said by another observer to be found in the urine, but this could never afterwards be confirmed. Marked anemia, red corpuscles 2,000,000, and hemoglobin 40 to 50 per cent. Great prostration. Weight reduced from a normal of about 150 pounds to 120 pounds. Considerable proteinuria. Since last autumn the patient has gradually improved, and at present, March 1, 1916, his weight has become practically normal; red corpuscles 4,000,000. He has now no acute pain in the back or legs, but sometimes slight aching and stiffness. He can walk some distance, but still has a feeling of weakness. Protein excretion still marked, hyaline and a few granular casts, not much improvement of vision (diagnosis by a specialist, paralysis of optic nerve). X-ray findings both of spine and long bones have always been entirely normal.

The urine has been uniform, of normal appearance except for the exaggerated foam formation upon slight shaking, and with marked acid reaction (litmus). The total protein was weighed from time to time and has also been rather constant, fluctuating between 23 and 30 gm. daily.

[1] A discussion of the clinical features of this case will be published shortly by Dr. Schoff.

Heat Reactions.—When the urine at natural reaction is heated coagulation commences at about 55°, and proceeds rapidly as the temperature passes this point. There is no apparent tendency to decrease until the boiling point is almost reached (97° or 98°) when a slight lightening of the density is sometimes to be noticed. As the liquid begins to boil, further clearing is observed, and when boiling has continued for a few seconds nearly all the protein is redissolved. Upon cooling, the protein commences to reprecipitate as soon as the temperature has been lowered a few degrees, and finally becomes as dense as before; this can be repeated indefinitely. It is clear that we have to do with the so called Bence-Jones protein. In order to observe the above in typical form it is necessary to dilute the urine, which is best done by adding normal urine. If the dilution is made with water the protein will often redissolve badly unless a salt or, better, a few crystals of urea[2] are added. If the urine is heated at its natural concentration some of the protein will be precipitated in elastic clumps (they can be drawn out to form bundles of coarse threads), which tend to adhere to the sides of the vessel or float on the surface and do not again dissolve. These clumps have also been described by Magnus-Levy.[2] When the Bence-Jones urine is diluted the precipitate is fine and milky, going in and out of solution with ease.

The addition to the native urine of one drop of 50 per cent acetic acid to 10 cc. of urine, prevents the formation of the clumps and causes the solution at boiling and reprecipitation on cooling to be much more clearly manifested. That this may be associated with some such action as acid-albumin formation is suggested by the following experiment.

We heated in the water bath at 52° for 10 minutes urine to which had been added one drop of 20 per cent acetic acid for each 10 cc. In order to obtain a maximum surface and so insure prompt heating, the urine was divided among a number of test-tubes. No apparent change was produced.

The temperature of the bath was then raised to 56° for 10 minutes, with the result of a heavy white curdy precipitate. This was filtered off, and of this filtrate, A, one part was diluted with three parts normal urine and heated over the flame. This produced a heavy white opacity which largely disappears on boiling, and upon the addition of some urea almost entirely

[2] Magnus-Levy, A., *Z. physiol. Chem.*, 1900, xxx, 200.

disappears; in either case it reappears when the liquid cools, and again disappears upon boiling.

The remainder of the filtrate A was put back in the water bath at 60° for 10 minutes. Result, heavy white curdy precipitate. It was filtered, and of this filtrate, B, one part was diluted with three parts normal urine and heated over the flame. This produced a moderate white opacity, which disappeared almost entirely upon boiling and reappeared on cooling.

The remainder of the filtrate B was again heated in the water bath, this time at 64° for 12 minutes. Result, moderately heavy white curdy precipitate. This was filtered, and of this filtrate, C, a portion diluted as before was heated over the flame. This produced a slight opacity, which disappeared almost entirely upon boiling, and reappeared on cooling.

The remainder of filtrate C was heated in the bath at 68° for 15 minutes. Result, slight white curdy precipitate. This was filtered, and of this filtrate, D, a portion without dilution was heated over the flame. The result was a slight opalescence, part of which disappeared upon boiling and returned on cooling.[3]

The rest of filtrate D was heated in the bath at 72° for 15 minutes. Result, slight opalescence. The filtrate, which even after repeatedly passing through the filter remained opalescent, was heated without dilution over the flame. Result, no change.

Under the conditions of the above experiment there is a continued precipitation from the temperature at which the Bence-Jones protein commences to come out up to the coagulation point of serum protein. This may be explained either by assuming that the Bence-Jones protein is composed of various fractions with different coagulation points, at least with different rapidity of precipitation; or, on the other hand, that the continued action of the acid had resulted in gradual change of the order of acid-albumin formation. We therefore made the following experiment.

Four tubes containing, respectively, 10 cc. of urine, 10 cc. of urine + 1 drop of 20 per cent acetic acid, 10 cc. of urine + 2 drops of acid, and 10 cc. of urine + 3 drops of acid, and a control tube of 10 cc. of nephritic urine (rich in albumin but apparently free from Bence-Jones protein) + 1 drop of the above acid, were placed in a water bath at 60° for 1¼ hours. The Bence-Jones tubes commenced to precipitate in the order 2, 3, 1, 0 (referred to by the acid content), following each other at intervals of 15 seconds to 1 minute. Soon all showed a heavy white curdy precipitate, the tubes appear-

[3] In order to make a certain judgment with these slight degrees of opalescence, the tube of urine is brought to boiling and then, avoiding shaking, the lower end (2 cm.) is held under the cold water tap, which permits of easy comparison between the hot and cold fluid.

ing almost solid. The control tube clouded slightly, and finally showed a very slight flocculent precipitate. At the end of the time the contents of all of the tubes were filtered and the respective filtrates heated over the flame. The filtrate from Tube 0 showed only slight opalescence, which was hardly affected by boiling. The same was true of Tube 1, although this was slightly decreased by boiling. These two filtrates also gave only moderate reactions with the Roberts test. The filtrate from Tube 2 showed marked opalescence, which nearly all disappeared upon boiling. The filtrate from Tube 3 gave a much heavier opalescence amounting almost to opacity, and this also was nearly all redissolved upon boiling. In both of these the original density returned on cooling. The filtrate from the control tube gave a considerable precipitate which showed no tendency to redissolve upon boiling.

It seems evident, therefore, that heating the Bence-Jones protein with even very moderate quantities of acetic acid is capable of effecting a change in the nature of the protein such that the temperature at which it coagulates is raised; but this in no way interferes with its characteristic behavior in regard to going into solution by boiling and reprecipitating on cooling.

Salt Reactions.—At the natural reaction of the urine one volume of saturated solution of ammonium sulfate added to one volume of urine causes incomplete precipitation, while two volumes cause complete precipitation.

Saturation with magnesium sulfate causes a considerable but not complete precipitation. This is opposed to the findings of most other writers, but Ville and Derrien[4] found that saturation with magnesium sulfate precipitated all the protein. When the urine is strongly acidified with acetic acid all the protein is precipitated by adding three volumes of a saturated solution of magnesium sulfate to one volume of urine.

If the urine at natural reaction be saturated with sodium sulfate, a large but not complete precipitation occurs. If a twice cold saturated solution of sodium sulfate be made up with hot water and allowed to cool almost to room temperature, it will be some time before the excess crystallizes out; if before this takes place one volume of the solution is added to an equal volume of urine containing a considerable amount of acetic acid, the precipitation is complete. Ammonium sulfate has the tendency to remove the pigment from the urine along with the protein; but sodium sul-

[4] Ville, J., and Derrien, E., *Compt. rend. Soc. biol.*, 1907, lxii, 679.

fate has little action on the pigments, and the protein when washed is pure white. The difficulty mentioned by Boggs and Guthrie[5] in obtaining a pigment-free precipitate is thus avoided.

If the urine at natural reaction is saturated with sodium chloride a slight opalescence results, but half saturation produces no effect. However, if sufficient acetic acid is added, precipitation is complete. Thus:

Urine	100	parts	Gradual and
50 per cent acetic acid	8	"	incomplete
Saturated solution NaCl	108	"	precipitation.
Urine	100	"	Prompt and
50 per cent acetic acid	20	"	complete
Saturated solution NaCl	120	"	precipitation.

When the clear filtrate from this last precipitation is added to an equal volume of Bence-Jones urine only a slight precipitate is caused; but if more acetic acid is added heavy precipitation occurs at once. It would seem that a large amount of acid is fixed in breaking up some alkaline combination in which the protein is normally found, and that most salts precipitate it quantitatively only after this decomposition has been effected.

Spiegler's solution, Roberts' reagent, and acetic acid and ferrocyanide all cause immediate heavy precipitation when used with the native urine; and all are capable of indicating minute traces of this protein. This is of course what would be expected, and is mentioned only because of the fact found by Folin and Denis[6] that the Bence-Jones protein gave no precipitate with acetic acid and ferrocyanide except after long standing.

Acids.—Concentrated sulfuric, nitric, and hydrochloric acids added drop by drop cause a heavy white curdy precipitate, insoluble in moderate excess of the acid. A large excess causes solution by destruction, which is indicated by the change of color in the case of the sulfuric and nitric acids. Acetic acid added drop by drop may at first cause a very slight cloudiness (often entirely absent—nucleoprotein) which disappears upon adding a little more acid. Citric acid causes no cloudiness.

[5] Boggs, T. R., and Guthrie, C. G., *Am. J. Med. Sc.*, 1912, cxliv, 803.
[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 277.

Urine diluted with two parts of water and treated with a small amount of either of the three mineral acids mentioned gives a dense white precipitate which dissolves upon boiling and reprecipitates upon cooling.

This solvent power of hot acids furnishes a satisfactory means of distinguishing between normal serum- and Bence-Jones protein. To a test-tube containing a few cc. of the urine to be examined is added a drop of concentrated nitric acid. This produces a white cloud or white curdy precipitate according to the amount of protein present; if a heavy curd is formed the urine should be diluted with water. Upon shaking, the precipitate disappears. Continue adding the acid drop by drop, shaking between each addition, until the precipitate or cloud no longer dissolves, then add one or two drops more. The contents of the tube are now brought to boiling. If the precipitate is Bence-Jones protein it will quickly dissolve, and upon cooling reprecipitates with the same white appearance as before; and this solution and precipitation can be repeated. If, on the other hand, the precipitate is due to normal serum protein it does not dissolve on boiling, but assumes the form of discrete flocks of a dirty yellowish or brownish color, not affected by cooling and not resembling in any way the white Bence-Jones precipitate.

Precipitation with HCl has been recommended as a specific test for Bence-Jones protein in the urine by Bradshaw[7] who states that albumin does not give this reaction unless present in very large amounts. Using strong HCl he assigns the limit of sensitiveness to Bence-Jones protein as 0.05 per cent, but apparently did not ascertain the percentage of serum protein that can be present without reacting. By a number of trials we found that the best differentiation occurs when: (1) the percentage of proteins is small; (2) the acid is comparatively weak, about 1 to 20 or 25; (3) when dilute acid is used, because if strong acid is added there is likely to be an immediate, more or less irreversible precipitation with either protein at the point of contact; (4) the mixture of urine and acid must be allowed to stand for a definite time, since eventually either protein will come down more or less completely.

[7] Bradshaw, T. R., *Brit. Med. J.*, 1906, ii, 1442.

In accordance with these principles the following method of testing was adopted. The dilute hydrochloric acid used consisted of concentrated acid one part, water three parts. In making the test the tube was well shaken after each addition, and after the acid was added was allowed to stand 1 minute before observing.

	cc.	
Normal urine	5	No reaction.
Normal dog serum	0.25	
HCl (diluted)	1	
Normal urine	5	No reaction or doubtful.
Serum	0.5	
HCl (diluted)	1	
Normal urine	5	Doubtful or very faint.
Serum	1	
HCl (diluted)	1	
Normal urine	5	Very faint opalescence.
Serum	2	
HCl (diluted)	1	

Even the last test was so faint that it would probably be rejected; but calling it positive, and assuming 6.5 per cent as the total protein content of the dog serum, the least amount of serum protein which will react with this test is 0.13 gm. protein or 1.86 per cent.

Testing now with Bence-Jones protein:

	cc.	
Normal urine	5	Faint opalescence.
Bence-Jones urine (2.7 per cent)	0.25	
HCl (diluted)	1	
Normal urine	5	Well marked opalescence.
Bence-Jones urine (2.7 per cent)	0.5	
HCl (diluted)	1	

The first of these two tests is somewhat clearer than the last of the previous series. However, to be on the safe side, if the second test be taken as the limit then the content is 0.013 gm. protein; thus, urine with 0.23 per cent Bence-Jones protein will react in this test. It is evident that this reaction is at least eight

times more sensitive for the latter than for the blood proteins. The ring test as described by Bradshaw although far more sensitive is less specific.

Besides the above hydrochloric acid test, we have found that lead acetate and zinc sulfate each have in the presence of acetic acid and under certain conditions power of differential precipitation between Bence-Jones protein and serum proteins. However, this action seems to depend upon so many factors that we have not so far succeeded in working out a practicable method of using these salts to distinguish between the proteins.

From a review of these reactions, especially the conduct with heat, where there is nearly always failure to obtain a quite complete re-solution upon boiling, we are inclined to believe that associated with the Bence-Jones protein is a small amount of serum protein; this conclusion is supported by the fact that a few casts are usually to be found upon centrifugation.

Separation of the Protein.—To each 100 cc. of filtered urine are added 20 cc. of 50 per cent acetic acid, and 120 cc. of a twice cold saturated and still slightly warm solution of sodium sulfate. This is stirred, allowed to stand about 20 minutes, and filtered with suction, using hardened paper. When the surface is dry the precipitate is washed three times with a cold saturated solution of sodium sulfate well acidulated with acetic acid. As soon as dry from the last wash solution, but while still pasty, it is scraped off from the paper and rubbed up in a dish to a thin milky suspension with 95 per cent alcohol. This rubbing is lightly done and the suspension is kept very fluid so as to leave behind as much of the sulfate as possible. From time to time the milky fluid is poured off into centrifuge tubes, a fresh portion of alcohol added to the dish, and the rubbing continued, always pouring off and renewing the alcohol before the suspension becomes rich enough to give the suggestion of cream. In this way the protein is gradually removed from the dish, together with some sulfate. Toward the end of the process the contents of the dish will be found to feel distinctly gritty from the sulfate crystals, and the operation should then be interrupted and the remainder discarded. The tubes are then centrifuged, the supernatant liquid is poured off, the tubes are filled with absolute alcohol, thoroughly stirred, and again

centrifuged. This alcohol is poured off and the washing process repeated. Then the protein is washed in the same manner once with a mixture of equal parts of absolute alcohol and ether, then twice with ether; finally it is collected on a filter of hardened paper and allowed to dry. Upon slight rubbing this protein will crumble to a fine white mobile powder, non-hygroscopic, scarcely soluble in water, but readily soluble in water or Ringer's solution when these are made slightly alkaline by dilute NaOH. In order to remove the remaining sulfate the dry protein is suspended in a large proportion of slightly warm distilled water in centrifuge tubes, stirred for a few minutes, and centrifuged. The wash water is then decanted from the protein, the alkaline water or other solvent poured in, and solution effected by stirring. Before dissolving, the washing with distilled water may be repeated, but this we usually omitted; if it be desired to obtain a strictly salt-free product this method should be replaced by dialysis.

When weak acetic acid is slowly added to a dilute slightly alkaline solution of the protein, some precipitation occurs at neutrality, which, however, readily redissolves upon the addition of a little more acid.

In alkaline solution the protein (like the native urine made alkaline) does not give the regular heat reaction. A solution of the protein with a very slight excess of acetic acid gives the reactions already described for native urine except that it does not coagulate upon heating. This is due to the absence of salts, for if a little sodium chloride is added, or especially if one or two volumes of normal urine are added to one volume of the protein solution, the typical heat behavior is fully restored.[8] It is therefore safe to assume that no material denaturation has been caused by this method of preparation. This conclusion is fully confirmed by the biological results described later.

For the purpose of estimating the protein, the simple heat precipitation in centrifuge tubes as described by Folin and Denis[6] (but with the use of only a trace of acetic acid) is very convenient.

[8] For the purpose of demonstration the protein may simply be suspended in normal urine of acid reaction, which is then boiled; whereupon the suspended particles promptly go into solution, with, again, a reproduction of the native Bence-Jones urine.

Preparing a Protein-Free Urine.—For subsequent work we required, in addition to the protein, urine from the same subject from which the protein had been quantitatively removed. The filtrate from the sodium sulfate precipitation was not suitable because of the large content of acid and besides it often contains traces of protein. We were not successful in strictly quantitative removal of the protein by heat alone; even the method given by Magnus-Levy[2] always allowed enough protein to remain in the filtrate to react with the Spiegler, Roberts, and the ferrocyanide tests. The following gave a strictly protein-free filtrate.

A mixture of 50 cc. of Bence-Jones urine, 50 cc. of water, and 100 cc. of sodium sulfate solution (100 gm. of sodium sulfate crystals in 200 cc. of water) is heated in the water bath; as soon as coagulation begins it is stirred occasionally with the thermometer until the temperature reaches 96–98°. Then it is taken from the bath, four drops of concentrated HCl are added, and the mixture is stirred well. After standing a minute or so it is rapidly cooled by immersing the vessel in cold water until the temperature has been reduced to about 40°, and filtered through 589 paper. The clear filtrate gives no reaction with any of the above reagents.

We also found that the protein can be quantitatively removed without heating, by the use of salt and acid. 2 cc. of concentrated HCl in 100 cc. of H_2O are saturated with sodium chloride. An equal volume of urine is added, and the mixture stirred for a minute, and filtered. The clear filtrate is entirely free from protein. This salt-acid solution is of course essentially the Roberts reagent for albumin.

Anaphylaxis.—This protein is capable of causing active anaphylaxis, as the following experiments show.

Anaphylaxis Series I.

Sensitization.—Nov. 27. Twelve guinea pigs were injected subcutaneously. Nos. 1 to 6 each received 1 cc. of Bence-Jones urine (0.027 gm. of protein). Nos. 7 to 12 each received 1 cc. of a 2 per cent solution of protein in Ringer's solution (0.020 gm. of protein).

Dec. 23. All were injected; the injection was into the jugular vein except for No. 2 which was injected directly into the heart.

Animal No.	Intoxication. Dec. 23 (26 days).	Results.
1	Diluted Bence-Jones urine, 0.012 gm. protein.	Moderate anaphylaxis with complete recovery at end of 15 min.
2	" urine, 0.003 gm. protein.	Very slight anaphylaxis with recovery in 6 min.
3	" " 0.004 " "	Slight anaphylaxis with recovery in 11 min.
4	1 cc. Ringer's solution, 0.002 gm. protein.	No symptoms.
5	4 cc. Ringer's solution, 0.008 gm. protein.	Very slight anaphylaxis with recovery in 5 min.
6	1 cc. urine, 0.024 gm. protein.	Same as No. 5.
7	1 cc. diluted urine, 0.004 gm. protein.	Marked anaphylaxis with death in 7 min.
8	1 cc. diluted urine, 0.002 gm. protein.	Marked anaphylaxis with death in 9 min.
9	1 cc. Ringer's solution, 0.002 gm. protein.	Marked anaphylaxis with death in 8 min.
10	*	
11	1 cc. Ringer's solution, 0.002 gm. protein.	Moderate anaphylaxis with recovery in 13 min.
12	2 cc. Ringer's solution, 0.004 gm. protein.	Marked anaphylaxis with death in 5 min.

* Lost.

From Series I it will be seen that: (1) the native Bence-Jones urine is capable of provoking only slight sensitization, whether the intoxicant is more of the urine or whether it is isolated protein; (2) the isolated protein is capable of causing very much higher sensitization against both native urine and isolated protein; (3) the native urine will intoxicate a sensitized guinea pig; (4) the isolated protein will also do this. The intoxicating power of the

native urine and of the isolated protein is about the same, so obviously the protein as prepared by us has suffered no material denaturation.

An interesting fact developed by this series is the weak sensitizing action of the native urine. Apparently some factor exists which prevents the protein from exerting its normal power; yet does not interfere with the subsequent intoxication of an animal properly sensitized by isolated protein. The experiments described in Series II were undertaken to study this inhibiting factor.

Anaphylaxis Series II.

Sensitization.—Jan. 12. Six guinea pigs were injected subcutaneously. Nos. 1, 2, and 3 each received 1 cc. of Bence-Jones urine (0.026 gm. of protein). This urine had previously been heated at 55° for 1 hour. Nos. 4, 5, and 6 each received 4 cc. of the following solution: 0.1 gm. of Bence-Jones protein dissolved in 20 cc. of urine from which the protein had been removed as described, and then made slightly alkaline. Nos. 4, 5, and 6 therefore each received 0.020 gm. of protein.

Feb. 4. All were injected into the jugular.

Animal No.	Intoxication. Feb. 4 (23 days).	Results.
1	1 cc. of diluted Bence-Jones urine, 0.001 gm. protein.	No symptoms.
2	1 cc. of undiluted urine, 0.025 gm. protein.	Well marked anaphylaxis with death in 10 min.
3	0.5 cc. urine, 0.013 gm. protein.	Moderate anaphylaxis with recovery in ½ hr.
4	1 cc. diluted urine, 0.001 gm. protein.	Marked anaphylaxis with death in 5 min.
5	1 cc. diluted urine, 0.0005 gm. protein.	Same as No. 4.
6	1 cc. diluted urine, 0.00025 gm. protein.	Moderate anaphylaxis with recovery in ½ hr.

From Series II it is clear that the inhibiting factor is thermostabile at 55°; for the guinea pigs sensitized with the urine which had been heated at this temperature[9] showed about the same sen-

[9] The urine became slightly opalescent from beginning precipitation of protein, and was not filtered.

sitiveness, allowing for the larger intoxicating doses, as did the animals of Series I which were sensitized with unheated urine.

On the other hand, it is clear that the power of sensitization of the isolated protein when dissolved in the deproteinized urine is even greater than when the same protein is dissolved in Ringer's solution. The removal of the protein from the urine was effected by the first (sulfate and heat) method, described on page 290, and was undoubtedly quantitative within the limits of the tests there mentioned; therefore the greater sensitization cannot be attributed to the presence of protein unaccounted for. Evidently the inhibiting factor is present neither in the isolated protein nor in this protein-free filtrate; it is apparently destroyed by chemical manipulation or by heating to near the boiling point. We are at present unable to make any further statement regarding this matter.

The marked anaphylactic activity of this protein confirms the belief that we are dealing with a higher protein of individual biologic stamp and not with a degradation stage in the hydrolysis of any body protein.

Direct Toxicity.—While it has generally been assumed that Bence-Jones protein is not directly toxic, there seems to be in the literature no definite experimental evidence bearing on this question. We therefore tried the effect of a large dose. A dog was etherized, and received into the femoral vein 2 gm. per kilo of body weight of protein dissolved in slightly alkaline Ringer's solution. The dog remained etherized for about 2 hours, and during this entire time the blood pressure taken by cannula from the carotid artery and the respirations from the trachea were recorded by the continuous paper kymograph. From the femoral artery blood was taken at short intervals to test its coagulability. In no direction was there the slightest indication of toxic action; and the normal clotting of the blood speaks strongly against the presence of proteoses, which would have been in sufficient concentration to interfere with coagulation. This is quite in accord with the anaphylactic activity already described.

Digestibility of Bence-Jones Protein.—This protein in the circulation of the patient is, like the plasma proteins, protected from digestion; but the isolated substance, evidently undenatured, is readily attacked by either pepsin or trypsin.

In one of two flasks, each containing a little less than 100 cc. of water, were placed 1 gm. of protein and 0.1 gm. of Gruebler trypsin; in the other flask 1 gm. of protein and 0.1 gm. of pepsin. The flasks were then brought to the proper respective reactions, made up to 100 cc. volume, a small quantity of toluene was added, well shaken, and placed in the incubator at 39°. After 48 hours in the incubator 25 cc. were withdrawn from each flask, 225 cc. of absolute alcohol added to each, and the whole was allowed to stand about an hour. The pepsin digestion gave at once strong opalescence followed by a flocculent precipitate, which weighed 0.042 gm. The filtrate from this precipitate was well diluted with water, boiled down to drive off the alcohol, and then gave a strong biuret reaction, showing that substantial digestion had occurred. The trypsin digestion was treated in the same way. Weight of precipitate, 0.031 gm. filtrate as above.

The flasks were returned to the incubator for another 48 hours, when 25 cc. portions were taken as before. The precipitate from each of these portions was found to be only 3 mg. less than after the first period, and the digestions were not carried any further. It would appear therefore that while nearly all the protein yields readily, a small fraction resists digestion with considerable tenacity.

As control a 1 per cent solution of protein was precipitated without any digestion; the filtrate did not give a biuret reaction; so that at least within the limits of this test all undigested protein is thrown down by alcohol. As further control another trypsin digestion was set up with the same proportions as before, but was boiled 15 minutes before placing in the incubator. After 48 hours the filtrate from the alcohol precipitate gave no biuret reaction.

If the Bence-Jones protein is to be regarded as foreign to the tissues of the subject, we should by the Abderhalden hypothesis expect that a special ferment would soon be aroused to destroy it in the body; and certainly there would seem to be nothing in the nature of the substance itself to resist such a fate. Yet instead of being digested large quantities of it are excreted in its native condition, apparently free from any hydrolysis; and this can continue indefinitely. Under such circumstances we must dismiss the idea of a protective ferment.

Abderhalden and Rostoski[10] produced by several injections of Bence-Jones protein in rabbits precipitins which reacted not only with this protein but also with human serum and the proteins prepared from it. Hopkins and Savory[11] did not obtain precipitins (which they explain as probably due to injecting insufficient amounts of protein), but they state that

[10] Abderhalden, E., and Rostoski, O., *Z. physiol. Chem.*, 1905, xlvi, 125.
[11] Hopkins, T. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.

anti-human serum gave a slight but definite reaction with solutions of Bence-Jones protein. Boggs and Guthrie,[5] by injecting solutions of Bence-Jones protein, produced in rabbits weak precipitins which reacted both to this protein and to blood proteins. In this connection it is interesting to note that Magnus-Levy[2] states that the character of the excretion of his patient slowly changed, becoming much less soluble on boiling than in the early stages of the disease, and finally behaved like ordinary blood protein. Yet he was satisfied that it was really the same substance because by changing the physical conditions of the test, as by the addition of salts or urea, the solubility at 100° returned.

From every point of view it is clear that this substance in our case as in others is a higher protein, a human product closely related to the normal blood proteins.

Our next paper will consider more fully the protein in the body and its elimination.

We desire to thank Dr. R. M. Pearce for his assistance with the anaphylaxis experiments; also Drs. J. E. Sweet and M. M. Peet for performing the experiment concerning the direct toxicity of the protein.

THE UREASE CONTENT OF CERTAIN BEANS, WITH SPECIAL REFERENCE TO THE JACK BEAN.*

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Urease has been found extensively in various bacteria, fungi, and higher plants. Takeuchi's discovery of urease in the soy bean[1] was followed by an investigation for urease in a number of the higher plant seeds by Kiesel[2] and later by Zemplén.[3] Falk[4] found urease in castor beans. Annett[5] examined a number of different varieties of the soy bean, finding the urease content about the same, while he also examined several Indian seeds for the presence of this enzyme. He made the interesting observation that the sword bean contained much more urease than the soy bean. Recently Benjamin[6] has noted the occurrence of urease in various nodules of the legumes and other plant parts.

Annett in speaking of the sword bean designates it *Canavalia ensiformis*. This designation properly belongs to the jack bean, a native of the West Indies and adjacent mainland, while the sword bean indigenous to India is properly designated *Canavalia gladiata*. These two beans have been much confused, and it is not quite clear with which bean this author has worked.[7]

In an examination of certain beans[8] we have found that the

* This paper was presented in abstract at the meeting of the American Society of Biological Chemists, December, 1915.

[1] Takeuchi, T., *J. College of Agric.*, Tokyo, 1909, i, 1.

[2] Kiesel, A., *Z. physiol. Chem.*, 1911, lxxv, 169.

[3] Zemplén, G., *Z. physiol. Chem.*, 1912, lxxix, 229.

[4] Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 292.

[5] Annett, H. E., *Biochem. J.*, 1914, viii, 449.

[6] Benjamin, M. S., *Proc. Roy. Soc., New South Wales*, 1915, xlix, 78.

[7] For a discussion of the jack and sword beans see Piper, C. V., *U. S. Dept. Agric., Bureau of Plant Industry, Circular 110*, 1913.

[8] We desire to thank Dr. Piper of the U. S. Dept. of Agriculture for kindly furnishing us with most of the beans used in this work.

jack bean contains a very much larger quantity of urease than the sword or soy beans, and in fact shows a greater enzymatic activity toward urea than any other source of the enzyme. The urease of this bean is also apparently as specific for urea as that of the soy bean.

Test of Beans for Urease.

The test for urease in the various beans was performed as follows: The beans were finely ground in a coffee mill, and one part of the powder was treated with ten parts of distilled water and allowed to stand for 1 hour with occasional agitation. The suspension was then centrifuged, and the clear or slightly opalescent fluid siphoned off. Two 5 cc. portions of each of these extracts were placed in two flasks of 200 cc. capacity. 10 cc. of a 1 per cent solution of urea were added to one of the flasks, the other serving as a control. The fluid in each flask was made up to 100 cc., toluene was added to prevent bacterial growth, and the flasks were tightly stoppered and allowed to remain at room temperature. At the end of 20 hours the solutions were titrated with 0.1 N hydrochloric acid and methyl orange. Table I shows the results obtained.

TABLE I.

Botanical name.	Common name.	0.1 N HCl.	
		Test solution.	Control solution.
		cc.	cc.
Phaseolus calcaratus.....	Rice bean.	1.10	1.00
" *angularis*.....	Adsuki "	1.20	1.10
" *aconitifolius*....	Moth "	1.50	1.30
" *mungo*.....	Mung "	1.05	1.00
" *aureus*.....	Urd "	5.30	1.00
Stizolabium deeringianum..	Early Florida velvet bean.	1.60	1.50
	Chinese velvet bean.	1.60	1.60
Vigna sinensis.....	Cow pea.	1.10	1.10
Dolichos lablab.....	Hyacinth bean.	1.30	1.20
Lupinus albus.....	White lupine.	29.50	1.30
Dolichos biflorus.....	Horse gram.	32.80	1.00
Glycine hispida.....	Soy bean.	33.00	1.80
Canavalia gladiata.....	Sword "	33.00	1.60
" *ensiformis*.....	Jack "	33.00	1.50

Six of the varieties of beans tested contained urease in sufficient amount demonstrable by this method. Three of these, white lupine,[3] soy bean,[1] and sword bean[6] have previously been shown to contain urease and are included for purposes of comparison. The samples which were negative by this test may contain traces of urease which would be detected by a more delicate or prolonged examination.

Relative Urease Activity.

The relative activity of the bean extracts containing urease was next tested. The extracts used throughout the work were always prepared as described above unless otherwise stated. 10 cc. of each extract were added to 50 cc. of 1 per cent urea solution, 5 cc. portions removed at various intervals, and the amount of decomposition was determined by titration. Table II gives the results expressed in cc. of 0.1 N hydrochloric acid. These figures are corrected for the alkalinity of the bean extracts.

TABLE II.

Time.	0.1 N HCl.					
	Phaseolus aureus.	*Lupinus albus.*	*Dolichos biflorus.*	*Glycine hispida.*	*Canavalia gladiata.*	*Canavalia ensiformis.*
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
¼						13.70
⅓	0.20	0.20	1.20	4.10	10.70	13.70
1		0.45	2.15	6.30	13.45	13.60
1½	0.30	0.65	2.90	7.80	13.50	13.60
2½	0.40	0.90	4.00	11.10	13.50	13.65
18	0.90	3.40	11.60	13.60	13.40	13.60

Assuming that the enzyme concentration multiplied by the time is a constant, as has been shown for soy bean urease,[9] we see that the jack bean contains over fifteen times, and the sword bean about five times as much urease as the soy bean, while the *Dolichos biflorus* contains about $\frac{1}{5}$, the white lupine about $\frac{1}{50}$, and

[9] Marshall, E. K., Jr., *J. Biol. Chem.*, 1914, xvii, 351. Van Slyke, D. D., and Cullen, G. E., *ibid.*, 1914, xix, 141.

the *Phaseolus aureus* about $\frac{1}{350}$ as much urease as the soy bean. These figures, of course, are very rough but indicate the great variation in urease content of the beans.

Inasmuch as the jack bean contains so much more urease than any of the others which have received investigation, further studies on this bean were made. Tables III and IV show the relative activity of the jack, sword, and soy beans. Extracts were prepared as usual and suspensions of the bean powders were also used. The two methods gave the same results, which fact is in favor of the view that the urease of each is practically completely extracted by the method employed.

The question of complete extraction was also tested directly by making an extract in the usual way, and then a second for a much longer time after rapidly washing the residue once from adherent remains of the first extract. The second extracts showed very little activity. An attempt was made to precipitate a part of the proteins by adding one-tenth volume of 0.1 N HCl to the extracts and filtering. This does not decrease the activity of the soy bean extract very much and was previously used by one of us. But with the sword bean and the jack bean extracts considerable loss of activity results.

In Table III 5 cc. of the extracts were added to 50 cc. of a 1 per cent urea solution, and 5 cc. portions of the mixture titrated at varying time intervals. In Table IV the experiment was similar to that of Table III except that 2 cc. portions of the extracts were used instead of 5.

TABLE III.

Time.	0.1 N HCl.								
	Soy bean extract.			Sword bean extract.			Jack bean extract.		
	Original.	Centrifuged.	Acidified.	Original.	Centrifuged.	Acidified.	Original.	Centrifuged.	Acidified.
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
$\frac{3}{4}$	3.1	3.0	2.5	7.9	7.8	3.7	15.65	15.6	10.2
$1\frac{1}{2}$	5.2	5.3	4.1	12.1	12.0	5.4	15.7	15.6	15.3
$2\frac{1}{2}$	7.3	7.4	6.0	15.3	15.0	6.8	15.6	15.6	15.45

TABLE IV.

Time.	0.1 N HCl.					
	Soy bean extract.		Sword bean extract.		Jack bean extract.	
	Original.	Acidified and filtered.	Original.	Acidified and filtered.	Original.	Acidified and filtered.
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
½	1.50	1.30	3.70	2.00	8.60	4.40
1	2.30	2.20	6.20	3.20	14.00	7.00
1½	3.10	3.10	8.20	4.10	15.70	8.00

The total amount of solids present in the extracts was determined by evaporating 10 cc. portions to dryness in weighed porcelain dishes, drying over sulfuric acid *in vacuo*, and weighing the residues. The averages of three determinations on different samples were, in mg. of solids per 1 cc.:

	mg.
Jack bean	26
Sword bean	24
Soy bean	41
Acidulated extract of soy bean	30

The jack bean extract contains less solid material than either the aqueous or acidulated extract of the soy bean.

Specificity of Jack Bean Extract for Urea.

The action of the extract was tested on a number of nitrogenous substances. *Ereptone* was used as representing a mixture of amino-acids and polypeptides which occur in the body. 0.200 gm. was dissolved in 25 cc. of water and 10 cc. of the extract were added. *Casein*: 0.050 gm. was treated with 15 cc. of water, and 10 cc. of the extract; 0.050 gm. of *hippuric acid* and 0.050 gm. of *leucylglycine* were each treated with 15 cc. of water and 5 cc. of the extract. 0.025 gm. of *phenylalanine uramino-acid* was dissolved in 10 cc. of water, and 10 cc. of extract were added. The solutions were all made slightly alkaline to sodium alizarin sulfonate with 0.1 N sodium hydroxide. An amino-acid determination by Van Slyke's method was made on 2 cc. portions of each solution at once, and again the next morning. Table V gives the results in amounts of nitrogen gas obtained.

TABLE V.

Substance.	Initial reading.	Temperature.	Pressure.	Final reading.	Temperature.	Pressure.	Time between initial and final readings.
	cc.	*°C.*	*mm.*	*cc.*	*°C.*	*mm.*	*hrs.*
Ereptone*	1.58	19.5	760	1.57	19.5	762	18
	1.55						
Casein	0.50	26.5	763	0.50	24	761	13
	0.48			0.59			
Hippuric acid	0.44	28	757	0.44	26	758	19
	0.43			0.42			
Leucylglycine	1.20	28.5	757	1.18	26	758	19
	1.18						
Phenylalanine uramino-acid	0.80	28	760	0.88	28	763	20
Jack bean extract alone	1.05	28	760	0.90	28	763	18
				1.14			

* Ammonia determinations on 5 cc. portions of the ereptone solution gave 1.10 cc. 0.02 N ammonia immediately and 1.20 cc. 0.02 N 18 hours later.

Table VI gives the results obtained on the action of the jack bean extract upon several other nitrogenous compounds occurring in animal fluids. 0.050 gm. of each substance was treated with 15 cc. of water, 10 cc. of jack bean extract were added, and

TABLE VI.

	Solution.	Control.
	0.02 N HCl required.	
	cc.	*cc.*
Jack bean extract alone	0.20	
Uric acid	0.20	
Creatinine	0.30	
Creatine	0.70	0.90
Adenine	0.25	0.20
Guanine	0.40	0.44
Glucosamine	1.30	1.30
Alloxantine	0.80	0.90
Betaine	0.25	
Tyrosine	0.80	1.00
Cystine	0.20	

the mixture was made just alkaline to sodium alizarin sulfonate. This solution was allowed to stand for 16 to 20 hours, and the ammonia determined by aeration. In cases where the amount of ammonia was greater than that contained in the control sample of 10 cc. of the extract, a second control was carried out by allowing the substance and 10 cc. of the extract to stand the same length of time and mixing just before aeration.

Preparation of the Enzyme in Solid Form by the Use of Organic Solvents.

The acetone method used by Van Slyke and Cullen[10] for the soy bean urease as well as other precipitants such as alcohol, mixtures of alcohol with ether, benzene, or ligroin were found to yield a dry powder of about the same activity per unit of weight as the original extract. 10 cc. portions of the jack bean extract, prepared by digesting one part of ground beans with ten parts of water and centrifuging, were poured into 100 cc. of the precipitant used. The acetone and mixture of two volumes of ether and one of alcohol gave the better yields (about 0.4 gm.) and also preparations of slightly greater activity. Tables VII and VIII show the comparative activity compared to the jack bean extract. In every case 25 cc. of a 1 per cent urea solution were added to the powder dissolved in 2 cc. of water, and 5 cc. portions titrated at varying time intervals.

TABLE VII.

Precipitant.	Weight of dried precipitate.	Time.			
		½ hr.	1 hr.	1½ hrs.	2 hrs.
		0.1 N HCl required.			
	mg.	*cc.*	*cc.*	*cc.*	*cc.*
Acetone	10	3.85	6.05	7.70	8.90
2 volumes of ether + 1 volume of alcohol	45.9	9.60	14.35	15.35	15.35
1 volume of benzene + 1 volume of alcohol	45.5	8.08	11.65	14.00	15.25
Jack bean* extract	45.0	9.85	14.20	15.25	15.30

[10] Van Slyke and Cullen, *J. Biol. Chem.*, 1914, xix, 211.

TABLE VIII.

Precipitant.	Weight of dried precipitate.	Time.	
		½ hr.	1 hr.
		0.1 N HCl required.	
	mg.	*cc.*	*cc.*
Acetone	49.5	12.80	15.45
Alcohol	48.7	11.00	15.40
1 volume of ligroin + 1 volume of alcohol	48.0	7.60	10.50
2 volumes of ether + 1 volume of alcohol	46.3	12.10	15.45
Jack bean* extract	57.7	12.40	15.45

* Jack bean extract was added in a volume sufficient to contain this much dried residue.

The urease of the jack bean should prove useful especially in procedures involving the rapid removal of urea from a solution in which but little contaminating foreign material must be added. The extract obtained by digestion with water and centrifuging or some solid preparation can, of course, be used for the estimation of urea in urine directly by titration,[11] and a much shorter time is required than with the use of soy bean extract where turbidity and masking of the end-point preclude the use of excessive amounts of the extract. The results in Table IX show that with human urine or animal urines diluted to contain 2.5 per cent or less urea, the conversion is complete in ½ hour at ordinary room temperature. The determinations were carried out using 5 cc. portions of urine, and 2 cc. of the jack bean extract. The results are expressed in cc. of 0.1 N acid required.

TABLE IX.

No.	Time.		
	½ hr.	1 hr.	20 hrs.
	0.1 N HCl.		
	cc.	*cc.*	*cc.*
1	36.00	35.90	35.90
2	39.30	39.00	38.90
3	41.70	41.60	41.40
4	28.60	28.45	28.60

[11] Marshall, *J. Biol. Chem.*, 1913, xiv, 283.

The extract can also be used for the removal of urea preparatory to estimating amino-acids in urine by Van Slyke's[12] method, instead of soy bean extract as suggested by thi author. A smaller amount of extract can be used, and the same time, giving a smaller blank, or the same amount of extract will require much less time.

SUMMARY.

Several varieties of beans have been examined for urease, and its presence or absence noted. The jack bean (*Canavalia ensiformis*) contains about fifteen times as much urease as the soy bean, while the extract from the jack bean contains much less solid residue than that from the soy bean. The urease of the jack bean as far as investigated appears to be as specific for urea as that of the soy bean. Suggestions are made wherein the urease of the jack bean may prove useful in chemical procedures.

[12] Van Slyke, *J. Biol. Chem.*, 1913–14, xvi, 125.

NUTRITION INVESTIGATIONS UPON COTTONSEED MEAL. I.

By ANNA E. RICHARDSON and HELEN S. GREEN.

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(Received for publication, April 24, 1916.)

Whether or not cottonseed meal can safely and advantageously be utilized for human consumption is a matter which up to this time has received little attention. Several metabolism experiments have been carried out to determine the coefficient of digestibility of cottonseed meal,[1] and rabbits and pigs have been used in studying the question of the toxicity of cottonseed meal and its extracts.[2] In considering the broad question of the nutritive value of cottonseed meal, which must necessarily include the study of its efficiency in promoting growth, development, and reproduction, conclusions based upon the results of a metabolism experiment which occupies but a few days, or deductions from the fatal results of feeding excessive amounts, or extracts from excessive amounts of cottonseed meal, cannot alone be accepted as conclusive.

In this paper we present a preliminary report on the efficiency of cottonseed meal as a food for promoting the growth, development, and reproduction of the albino rat. The general plan of the investigation was suggested by the work of Osborne and Mendel, and the technique employed follows the methods observed by the authors on their visit to the Connecticut Agricultural Experiment Station.[3]

[1] Fraps, G. S., *Texas Agric. Exp. Station, Bull. 128*, 1910. Rather, J. B., *Texas Agric. Exp. Station, Bull. 163*, 1913.

[2] Withers, W. A., and Ray, J. B., *J. Biol. Chem.*, 1913, xiv, 53. Withers, W. A., and Brewster, J. F., *J. Biol. Chem.*, 1913, xv, 161. Crawford, A. C., *J. Pharm. and Exp. Therap.*, 1909–10, i, 519.

[3] We wish to thank Dr. Osborne and Miss Ferry for their advice and help as to the best method of conducting this work.

The rats used in the experiment were bred in Texas from Massachusetts stock. Through a careful process of elimination a sturdy resistant stock has been obtained. During the summer of 1915 the excessive heat in Texas appeared to have a slight retarding influence upon the growth not only of the experimental but also of the control animals.

Our plan of investigation includes the consideration of the following points.

1. Can animals live and thrive on a diet of which cottonseed meal comprises 33 to 50 per cent?
2. Can animals live and thrive on a diet in which cottonseed meal furnishes the only source of protein?
3. What nutritive factors are lacking in cottonseed meal to make it an efficient food *per se*?
4. Can cottonseed meal be combined with any foodstuffs to make an efficient diet?
5. Does cottonseed meal contain a toxic substance; if so, how does it act?

This paper reports the data of the first year's experiments, whose value will be more definitely determined by the results obtained from a longer period of experimentation.

In these experiments the control animals have been fed either an ordinary mixed diet consisting of dog biscuit,[4] milk, grain, green vegetables, and occasionally meat, or the following diet, which was recommended to us by Osborne and Mendel.

	per cent
Whole milk powder[5]	60
Starch	12
Lard	28

Cottonseed meal or flour, supplemented by other substances, was used in all the experimental diets. In the percentages of the protein content of these diets, we have imitated the control whole milk powder diet which supplies 25 per cent protein. All experimental diets are made up in paste form with 28 to 30 per

[4] Special dog biscuit manufactured by Potter and Wrightington, after the formula of Dr. Castle, of Bussey Institute. This biscuit was recommended by Osborne and Mendel for feeding stock rats.

[5] Whole milk powder containing 27.5 per cent butter fat, manufactured by Merrell-Soule, Syracuse, New York.

cent fat. We have used two cottonseed products, cottonseed meal and Allison cottonseed flour. The flour has been made by refining commercial cottonseed meal and contains from 50 to 58 per cent protein and about 12 per cent fat. It has been deprived of the greater part of the lint, hulls, and resin, by the heating, bolting, etc., of the Baumgarten process.[6] The ordinary commercial cottonseed meal has been passed through a twenty mesh sieve to remove the lint and hulls to a uniform degree. This meal contains more hulls, resin, and coloring matter than the bolted flour. Both of these forms have been employed to test any nutritive advantage, or difference in degree of possible toxicity.

In the experiments the following diets have been used.

		per cent
1.	Cottonseed flour	50
	Starch	22
	Lard	28
3A.	Cottonseed flour	50
	Starch	22
	Butter fat	12
	Lard	16
5.	Ether-extracted meal	50
	Starch	22
	Lard	28

		per cent
2.	Cottonseed flour	33
	Whole wheat flour[7]	37
	Lard	30
4.	Cottonseed flour	70
	Lard	30
7.	Cottonseed flour	50
	Protein-free milk[8]	22
	Butter fat	12
	Lard	16

[6] Manufactured by G. A. Baumgarten, Schulenburg Oil Mills, Schulenburg, Texas.

[7] Flour made from the whole crushed wheat, ground in the mill twice, and sifted through a twenty mesh sieve.

[8] For a short period protein-free milk was prepared according to the method of Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911. Later it was found more convenient to use Merrell-Soule skim milk powder instead of fresh milk. After neutralizing the clear filtrate, the final step in the process described by these investigators, the liquid was again filtered to remove the small amount of protein precipitated by the neutralization. This preparation contains a smaller percentage of nitrogen than the original Osborne and Mendel preparations. These results were corroborated by the work of Mitchell, H. H., and Nelson, R. A., *J. Biol. Chem.*, 1915, xxiii, 461.

		per cent
8.	Sifted cottonseed meal..	50
	Starch.....	22
	Lard.....	28

		per cent
13.	Cottonseed flour.....	45
	Whole milk powder.....	17
	Starch.....	10
	Lard.....	28

14.	Cottonseed flour.....	50
	Protein-free milk.....	22
	Lard.....	28

Do Animals Live and Thrive on Cottonseed Meal?

At the present time animals are alive after eating cottonseed meal diets for 310 to 345 days. These diets contained from 33 to 50 per cent cottonseed flour. The oldest animals have been on Diets 1 and 7 containing 50 per cent cottonseed flour. Others have received 50 per cent cottonseed flour in Diet 3 A and 33 per cent in Diet 2. While the animals on these diets have manifested a slower rate of growth than the control animals, yet the fact that rats are still alive after 310 to 345 days, approximately one-third of the extreme span of a rat's life,[9] on a diet which contains 33 per cent or more of cottonseed meal, is extremely important and one to be referred to in connection with the possible toxicity of cottonseed meal.

Cottonseed Meal as the Sole Source of Protein.

With cottonseed flour as the sole source of protein, rats are still alive after 340 days. On Diet 1, in which the flour furnished 25 per cent protein, there have been growth, maintenance, and well nourished appearance in several rats for a period of about 135 days. Several rats which were continued beyond 135 days on Diet 1 steadily lost in weight, became greatly emaciated, and died after 152 to 157 days. Others recovered when additions were made to the diets. Rat 49, an especially sturdy animal, upon indicating failure to grow or maintain body weight on Diet 1 after 210 days was changed to Diet 3 A, which differed from Diet 1 only in 12 per cent butter fat content. With this addition of butter fat, recovery was almost complete. After 38 days failure was again evident, but an immediate gain in weight occurred

[9] Donaldson, H. H., Boas Anniversary Volume, New York, 1906, 5.

when the animal was supplied with Diet 7, which contains protein-free milk in addition to butter fat. Rat 36 on Diet 1 grew steadily for 137 days. At this point failure appeared and the animal's weight dropped so rapidly that the immediate substitution of an efficient diet was imperative. On Diet 7, containing butter fat and protein-free milk, this animal has recovered rapidly and reached practically normal full size at the age of 315 days.

Rats on Diet 4 made up of 70 per cent cottonseed flour and 30 per cent lard, have grown normally for 90 days. From this point they have grown very slowly or have maintained body weight and good appearance for 70 days longer. This diet is deficient in carbohydrate, some of the necessary inorganic constituents,[10] and possibly other substances essential in a complete diet (Chart 4). On Diet 4, Rat 44, although his resistance to such an adverse diet was not as great as that of other rats, demonstrated in a remarkable manner that this diet is sufficient for growth and maintenance for a considerable time. This animal on Diet 4, after reaching the age of 70 days, weight 83 gm., became extremely emaciated and too weak to stand. The animal's fur was rough and stood on end; feet, tail, and ears were colorless and transparent; and the eyes were swollen shut. At this point, when death seemed imminent, protein-free milk and butter fat were supplied as in Diet 7. Recovery was immediate and complete, normal growth was initiated, and after 42 days on this diet the rat was returned to 70 per cent cottonseed meal, on which it continued to grow for 77 days, maintained body weight for 52 days, and died at the age of 263 days, a well nourished, fat, fine-furred individual.

By referring to Chart 1 it appears that rats on 70 per cent cottonseed flour and 30 per cent lard have grown better than

[10] Ash analysis of Allison cottonseed flour:

	per cent
Inorganic salts	5.50
SiO_2	0.14
Cl	None.
SO_3	0.06
P_2O_5	2.57
K_2O	2.01
CaO	0.26
MgO	0.25
Na_2O	None.

Rats 111 and 50 on 50 per cent cottonseed flour, 28 per cent lard, and 22 per cent starch. In every case in which the diet showed a deficiency, the rats were recovered (Chart 2) by the addition of some substance other than protein to the diet. The fact that cottonseed flour has furnished the only source of protein for rats during 340 days and that rats have grown for 90 days and maintained body weight for 70 days more on a diet containing 70 per cent cottonseed flour and 30 per cent lard, is evidence that the protein of cottonseed flour is an available protein and is sufficient for growth.

What Nutritive Factors Are Lacking in Cottonseed Meal?

Rats which received 12 per cent butter fat in Diet 3 A have not grown at any greater rate than those on Diet 1, but growth has continued for a longer period, probably due to the growth-promoting factor contained in butter fat[11] (Chart 3). With Rats 37 and 38 on Diet 3 A steady growth was maintained for 185 and 188 days before failure and, in the former case, death occurred. Rat 38 grew steadily for 197 days and maintained body weight for 39 days more; at this point the growth was resumed after improving the mineral content of the diet[12] by the addition of protein-free milk.

The recovery of Rats 36 and 49 on Diet 7 has already been mentioned. Rat 45 was reduced to a degree of emaciation and a condition similar to that of Rat 44 on the same Diet 4. When protein-free milk and butter fat were added as in Diet 7, a sudden growth impulse was manifested which persisted for 26 days when the animal died of lung trouble while still growing. With Rat 38 after 224 days of growth and maintenance on Diet 3 A, additional growth was promoted by 22 per cent protein-free milk. Rat 40 on Diet 1 grew slowly, probably due to a food intake below normal. Later with a more normal intake growth became pronounced. Eventually failure of the diet became evident, and when 12 per

[11] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.

[12] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615. Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station, Research Bull. 17*, 1911. Osborne and Mendel, *J. Biol. Chem.*, 1913, xv, 311.

cent butter fat was added, a slight but not continuous recovery occurred. However, when protein-free milk was added, a decided, immediate, and continuous growth was manifested (Chart 2).

We have not fed a diet containing ideal inorganic constituents, as protein-free milk and butter fat, for a long enough period to offer any conclusions as to the efficiency of such a diet over a long period compared with the whole milk powder control diet, or an ordinary mixed diet in promoting growth and development, but after 90 days on Diet 7, containing 50 per cent cottonseed flour, 22 per cent protein-free milk, 12 per cent butter fat, and 16 per cent lard, a large number of rats have grown normally and apparently are in as good physical condition as the control animals of the same age. This evidence considered together with the remarkable manner in which Diet 7 has recovered all rats reduced to extreme emaciation and possible death on incomplete diets, and induced immediate growth, indicates the lack in cottonseed meal of some essential nutritive factor rather than the presence of a deleterious substance. Whether this is due to deficiency in the inorganic salts[10] or to the lack of some growth-promoting factor has not been determined. The diet containing cottonseed flour and protein-free milk without butter fat has not been continued long enough to justify definite conclusions, but after 90 days on a diet containing 50 per cent cottonseed flour, 22 per cent protein-free milk, and 28 per cent lard, several rats have attained normal weight for control animals of the same age.

Will Cottonseed Meal Combined with Some Common Food Material Make an Efficient Diet?

In endeavoring to find a possible combination of cottonseed meal with some common food which would prove adequate for growth and development, cottonseed meal was combined with milk powder.[13] On this, Diet 13, perfectly normal growth and development of twelve animals have been obtained (Chart 5). Of the six females in this group, five have borne one or more litters of young. Rat 89 at the age of 146 days bore eight young, and ate all but four which were adopted by a stock female and after weaning were given Diet 13. At the present age of 70 days they

[13] Whole milk powder, Merrell-Soule.

are growing as well as control animals. 31 days after the first litter, Rat 89 bore five young. Rat 125 at the age of 148 days bore five young, and 56 days later, six young; Rat 134 at the age of 128 days bore seven young; Rat 136 at the age of 133 days bore six young, and 56 days later, five young; Rat 91 at the age of 189 days bore four young. The young rats after weaning have lived on the same Diet 13, as their mothers and are normal at the ages of 60 to 70 days.

This again points to the absence of an active toxic substance, since the addition of even 17 per cent of as favorable a food as milk powder could scarcely counteract the harmful effects of 45 per cent cottonseed meal if it contained an active toxic substance.

No young animals have been obtained from rats on Diets 1, 2, 3 A, 4, 5, and 8, containing from 33 to 70 per cent cottonseed meal or flour. All these diets were deficient in necessary minerals which alone might account for the inability to reproduce,[14] as it has been demonstrated that diets which are lacking in necessary inorganic constituents but are otherwise adequate are inefficient for the reproduction of the albino rat. We are at this time testing the possibility of reproduction on diets composed of cottonseed meal, butter fat, and essential inorganic salts furnished by protein-free milk, and we hope to report on this point later.[15]

Toxicity of Cottonseed Meal.

Animals fed on cottonseed meal extracted with ether according to the method of Withers[16] have shown no nutritional advantages over those rats fed on the unextracted meal. In fact, failure and death occurred much sooner on the former diet, 5, than on the latter, 8. Perhaps this is due to the absence through extraction of the ether-soluble, growth-essential substance present in butter fat which appears to be present in small quantities in unextracted meal, since in the case of Rat 78, after a period of 240 days on a diet of ether-extracted meal, during which time there

[14] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 615.

[15] Since this paper was started Rat 38, on Diet 7, has borne seven young, all in good condition.

[16] Withers, of the North Carolina Agricultural Experiment Station, believes that the ether extract of cottonseed meal contains a toxic substance which he calls *gossypol*.

was slight growth and later just maintenance, when butter fat was added to the diet pronounced growth was manifested.

In comparing the behavior of rats on cottonseed meal in Diet 8 and on the more highly milled flour in Diet 1, there is indication of a slight nutritive difference in the two products. Rat 86, Chart 1, on the ordinary meal, Diet 8, is still growing at a period beyond the time at which complete failure of Rat 50 on Diet 1 appeared. Chart 1 shows that while Rat 86 on Diet 8 has grown at a slower rate than Rats 50 or 111 on Diet 1, Rats 142 and 143, representative of animals of greater stamina, on Diet 8 are growing at a greater rate than any on Diet 1. This again suggests a greater amount of the growth-promoting substance associated with certain fats in the less highly milled product.

SUMMARY.

1. Albino rats are still alive after 310 to 345 days on diets containing 33 to 50 per cent cottonseed flour.

2. With 50 per cent cottonseed flour as the sole source of protein, mineral, and growth-promoting factors in Diet 1, rats have grown and maintained body weight for 135 days. With 50 per cent cottonseed flour as the sole source of protein rats have lived for 345 days. When cottonseed flour has been reinforced with protein-free milk to furnish adequate inorganic salts and butter fat containing a growth-promoting substance, rats which have failed on deficient diets have promptly recovered and exhibited a rate of growth nearly normal. Rat 38, thus recovered, has borne a litter of seven. Rats receiving 70 per cent cottonseed flour as the only source of protein, mineral, carbohydrate, and probable growth-promoting substances, have grown more normally for a period of 90 days, than the animals receiving 50 per cent cottonseed flour with the addition of 22 per cent carbohydrate as starch.

3. Rats receiving butter fat in a diet in which cottonseed flour is the only source of protein and mineral, show some nutritional advantage over rats not receiving butter fat in a similar diet, and have grown for a slightly longer period.

4. Rats which have failed on Diet 1 have recovered for a short period on Diet 3 A containing butter fat, but permanent recovery has occurred only when the inorganic salt content of the diet has been improved by the addition of protein-free milk as in Diet 7.

5. On a diet containing 45 per cent cottonseed flour, 17 per cent whole milk powder, 10 per cent starch, and 28 per cent lard, rats have grown and developed normally to the age of 6 months. Five females have borne one or more litters of young which are growing normally at the age of 60 to 70 days, on the same diet as the mothers.

6. Rats receiving cottonseed meal, containing more resin, fat, lint, and hulls than the flour, have shown nutritional advantage over those receiving cottonseed flour. This may be due to a greater amount of the growth-promoting factors in the meal than in the refined flour.

Our results indicate that cottonseed meal does not contain sufficient minerals for growth, is not actively toxic, contains efficient protein, and perhaps fat-soluble growth-promoting substances, similar to those of butter fat, but in less adequate quantities.

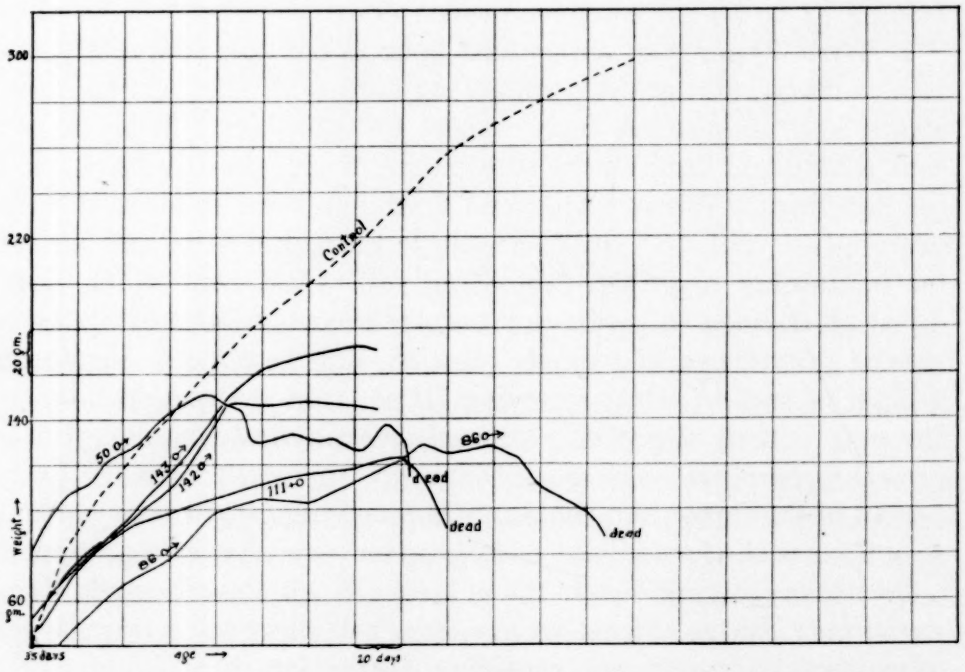

CHART 1. The growth of Rats 50 and 111 on refined cottonseed flour, Diet 1, and Rats 86, 142, and 143 on ordinary cottonseed meal, Diet 8. On the latter diet Rat 86 is growing at a period beyond the point at which rats, for example No. 50, have failed entirely on Diet 1. Rats 142 and 143, of sturdier stock than Rat 86, have grown almost normally for 110 days on Diet 8.

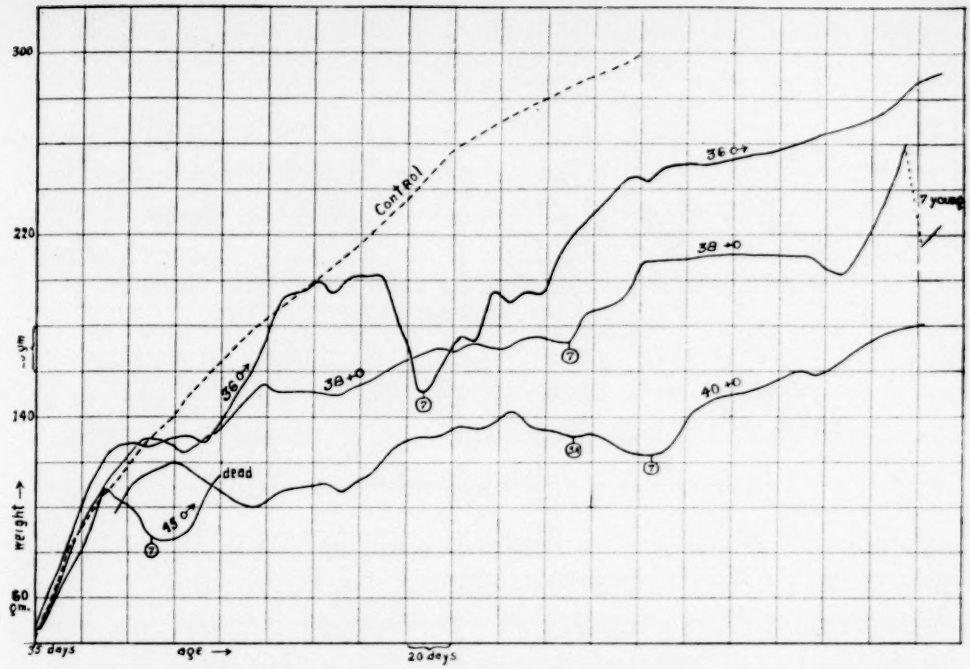

CHART 2. Rats which have failed on diets deficient in one or more essential nutritive factors have recovered on Diet 7, which supplies all of these factors. The point where figure 7 appears indicates when Diet 7 was given to each rat.

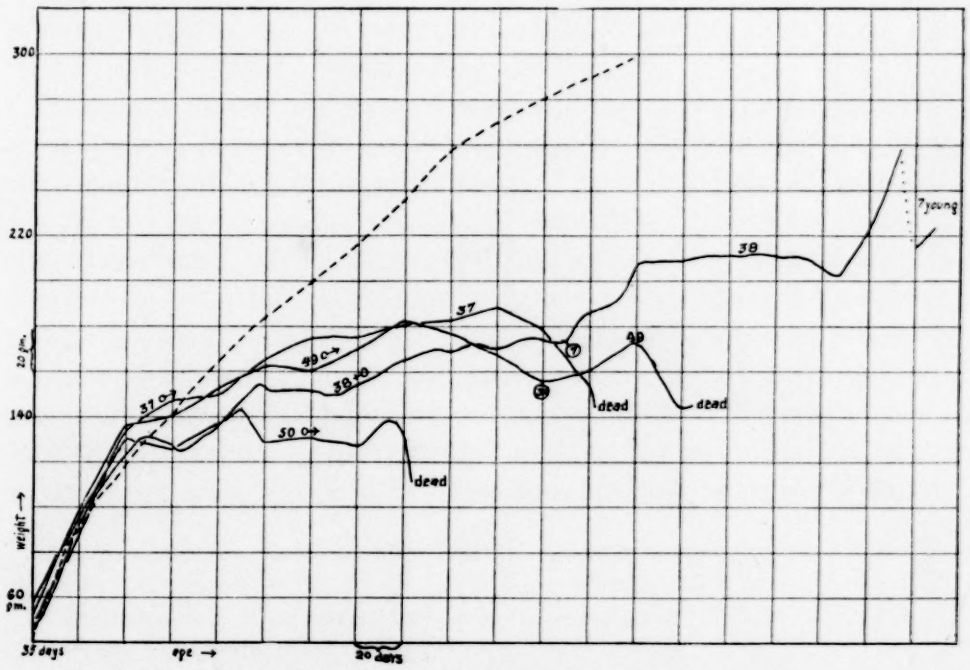

CHART 3. The efficiency of a cottonseed flour diet with butter fat, Diet 3 A, and without butter fat, Diet 1. Rats 38 and 37 on Diet 3 A continued to grow for 185 to 188 days, a longer period than Rats 49 and 50 on Diet 1 for 135 days. Note that Rat 38 was given further but not continuous growth by the addition of butter fat in Diet 3 A.

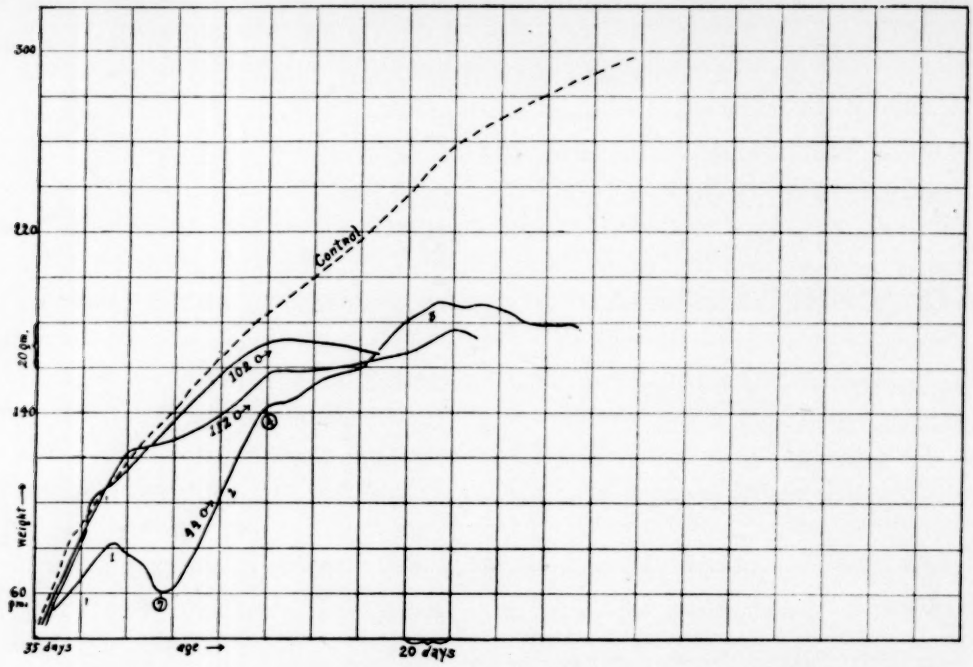

CHART 4. Rat 44 in the first period received 70 per cent cottonseed flour in Diet 4; in Period 2 recovered by Diet 7; in Period 3 returned to Diet 4, on which it survived a total of 170 days and died, a well nourished animal. Rats 102 and 112 have lived 150 days on Diet 4, and grown almost normally for 90 days.

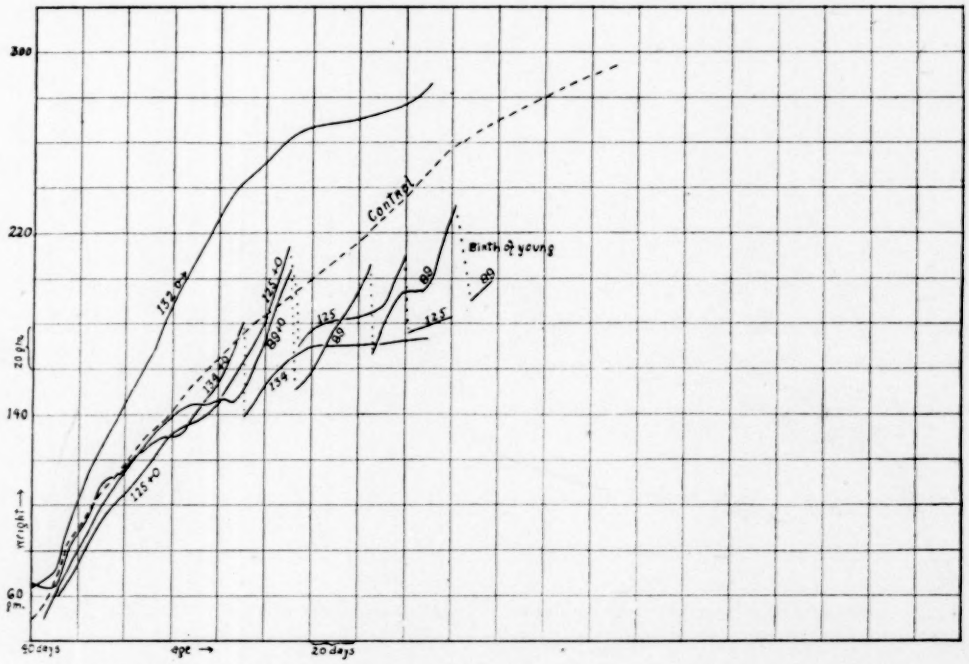

CHART 5. Rats 89, 125, 132, and 134 on Diet 13, cottonseed meal and whole milk powder, show a rate of growth apparently normal. Rats 89 and 125 each have produced two litters of young, and Rat 134 one litter, all of which are growing normally at the age of 60 to 70 days on Diet 13.

THE NINHYDRIN REACTION WITH AMINO-ACIDS AND AMMONIUM SALTS.*

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(Received for publication, April 22, 1916.)

Among the many biological reactions which have attracted attention during recent years, the one bearing the name of Abderhalden[1] has been prominent. The simple nature of the underlying idea, that a circulating foreign protein induces in the blood a corresponding and specific defensive ferment which accomplishes its hydrolysis, combined with the obvious importance of its applications, has rendered the reaction the object of numerous investigations. Both theory and results have been assailed from many directions and the reaction is still a matter of doubt, though evidence is steadily accumulating against it.[2]

The detection of the hydrolysis products of the circulating foreign protein is usually carried out by the dialysis method, and depends ultimately on the detection of these products after dialysis, by the now so called ninhydrin reaction. This latter reaction was discovered by Ruhemann[3] who found that α-amino-acids when warmed with triketohydrindene hydrate gave a fine blue color. β- and γ-amino-acids hardly reacted at all, and α-amino-acids when substituted on the amino or carboxyl group, gave a negative reaction. Abderhalden and Schmidt[4] confirmed and extended Ruhemann's observations, and, as far as is known, there is no amino-acid obtained from protein hydrolysis which does not

* A preliminary note on this and the succeeding communication was published by Harding, V. J., *Tr. Roy. Soc. Canada*, 1915, series 3, ix, 33.

[1] Abderhalden, E., *Handb. biochem. Arbeitsmethoden*, 1912, vi, 223; *Münch. med. Woch.*, 1912, lix, 1305, 1939, 2172.

[2] Van Slyke, D.D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377. Hulton, F., *J. Biol. Chem.*, 1916, xxv, 163.

[3] Ruhemann, S., *J. Chem. Soc.*, 1910, xcvii, 2030; 1911, xcix, 798.

[4] Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, 1911, lxxii, 37.

respond readily to the test. Proteins and their hydrolysis products were also found to give a positive reaction. One or two ammonium salts and a few bases were examined with negative results, and thus the test began to be regarded as characteristic of proteins and their hydrolysis products.

Herzfeld,[5] however, noticed that ammonium carbonate and ammonium oxalate when evaporated to dryness with triketohydrindene hydrate gave a positive reaction. It was, however, due to Neuberg[6] that the fact was discovered that the ninhydrin reaction with ammonium salts was a general one, thus contradicting the experiments and conclusions of Abderhalden and Schmidt. Neuberg also discovered the positive nature of the ninhydrin reaction with many organic bases and these results will be discussed in the succeeding paper.

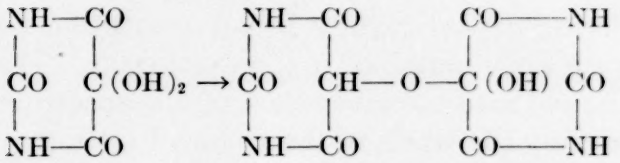
The results of Neuberg destroy at once the specific nature of the ninhydrin reaction, and we wish in this paper to discuss the probable cause of the discrepancies and endeavor to gain some insight into the reaction.

The Analogy between the Ninhydrin Reaction and the Formation of Murexide from Alloxan.

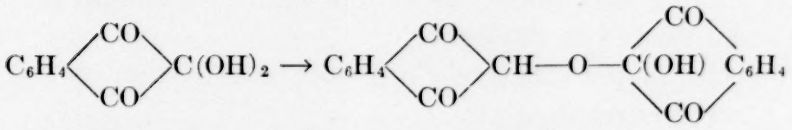
This analogy was pointed out by Ruhemann,[7] who showed conclusively that the blue coloring matter produced by heating amino-acids and triketohydrindene hydrate was similar in properties and in method of preparation to murexide. Basing his ideas on the constitution of murexide advanced by Piloty and Finckh,[8] and by Slimmer and Stieglitz,[9] and the analogy between triketohydrindene hydrate and alloxan, he prepared the coloring matter in the following way.

Alloxan and triketohydrindene hydrate on reduction with mild reducing agents yield alloxantin and hydrindantin respectively, to which he gave the following constitutions.

[5] Herzfeld, E., *Biochem. Z.*, 1914, lix, 249.
[6] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.
[7] Ruhemann, *J. Chem. Soc.*, 1911, xcix, 792, 1486.
[8] Piloty, O., and Finckh, K., *Ann. Chem.*, 1904, cccxxxiii, 22.
[9] Slimmer, M., and Stieglitz, J., *Am. Chem. J.*, 1904, xxxi, 661.

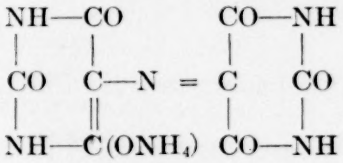


Alloxan. Alloxantin.

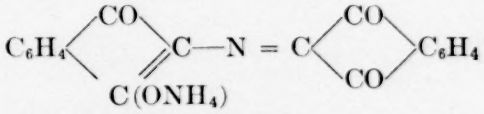


Triketohydrindene hydrate. Hydrindantin.

On warming with a mixture of ammonium carbonate and ammonium acetate these gave murexide and the ammonium salt of diketohydrindylidene-diketohydrindamine respectively.



Murexide.



Ammonium salt of diketohydrindylidene-diketohydrindamine.

The ammonium salt of diketohydrindylidene-diketohydrindamine prepared in this way through the intermediate hydrindantin was found to have a blue coloration identical with that formed by heating alanine and triketohydrindene hydrate, the alanine being oxidized to carbon dioxide and acetaldehyde. Triketohydrindene hydrate and glycine were shown to give formaldehyde, carbon dioxide, and the ammonium salt of diketohydrindylidene-diketohydrindamine. Strecker[10] had shown that alanine and alloxan, when heated together in aqueous solution, produced acetaldehyde, carbon dioxide, and murexide. Thus a close analogy between the two reactions was established.

The identity of the ammonium salt of diketohydrindylidene-diketohydrindamine was established by Ruhemann by means of analysis and chemical reactions. As a further means of identification, especially in dilute solution, we have observed three properties which can be used for the detection of the compound.

[10] Strecker, A., *Ann. Chem.*, 1862, cxxiii, 363.

1. The coloring matter gives a broad absorption band in the visible spectrum when examined in dilute solution. This band extends from the red into the green part of the spectrum, blocking out almost entirely the whole of the yellow.[11]

2. The blue color changes to a purple when viewed in artificial light, resembling very much the color of dilute potassium permanganate when viewed in daylight.

3. The color is quantitatively resistant to oxidation by the passage of a rapid current of air.

Prepared by either of the two methods—by the action of ammonium carbonate and acetate upon hydrindantin, or by the interaction of alanine and triketohydrindene hydrate—the coloring matter shows the same characteristics in dilute solution. It is also of interest to note that murexide gives a similar absorption band,[12] thus supporting the analogy drawn by Ruhemann.

The Interaction of Ammonium Salts and Triketohydrindene Hydrate.

It has just been stated that various contradictory results had been obtained by the earlier observers on the positive or negative nature of the ninhydrin reaction with ammonium salts. This we believe has been due to the use of ammonium salts of different concentrations. In our experiments we have used the pure ammonium salts obtainable from Kahlbaum or Schuchardt. Where these were not available we have taken a known weight of the pure acid, and added the theoretical amount of ammonia from a standardized solution of ammonium hydroxide. In this way we have made sure of the purity of our salts. The purity of the Kahlbaum or Schuchardt specimens was controlled by a determination of their ammonia content by means of Nesslerization.

The process of carrying out the ninhydrin reaction has been to take 1.0 cc. of ammonium salt and 1.0 cc. of 1 per cent ninhydrin solution, and heat them for 20 minutes in a rapidly boiling water bath.

[11] We have found, since writing the preliminary report, that the existence of this absorption band was first noticed by Deniges, G., *Bull. Soc. Pharm. Bordeaux*, 1914, liv, 49, of whose work we were unaware at the time.

[12] Compare Hartley, W. N., *J. Chem. Soc.*, 1887, li, 153.

The following table shows the results obtained:

TABLE I.

Salt.	Concentration.	Reaction.
	per cent	
Chloride	1.0	No reaction.
Nitrate	1.0	" "
Sulfate	1.0	" "
Hydrogen phosphate	1.0	Intense violet red; blue on dilution.
Sodium hydrogen phosphate	1.0	" " " " " "
Formate	0.92	Deep violet red.
Acetate	0.28	Violet.
Carbonate	1.0	Intense violet red; blue on dilution.
Succinate	1.0	Deep reddish violet.
Glycollate	1.0	Violet.
Lactate	0.8	"
Malate	0.2	"
Tartrate	1.0	Reddish violet; blue on dilution.
Citrate	1.0	Violet.
Oxalate	1.0	Deep reddish violet; blue on dilution.
Benzoate	1.0	Violet.
Thiocyanate	1.0	Deep reddish violet.

A glance at the table will show that all the ammonium salts tested, with the exception of those of the strong mineral acids, give a positive ninhydrin reaction. By increasing the concentration, however, even the salts of these acids can be made to show a positive test. Thus in a concentration of 5 per cent, ammonium sulfate gives a decided pinkish color. The chloride and the nitrate, however, require to be nearly saturated before responding, faintly but unmistakably, to the test, and it certainly seems significant that the salts of the strongest acids require to be used at the highest concentration before they show the ninhydrin reaction. Our own list contains seventeen ammonium salts: the two of Neuberg[6] contain nineteen other salts, including salts of many rare thio-acids, and together they include almost every type of acid, so that we feel justified in stating that the ninhydrin reaction is given by all ammonium salts, provided the concentration be sufficiently high. If, however, the concentration is very low

a negative reaction is obtained in all cases. Thus when the concentration of the ammonium salt is such that 1.0 cc. = 0.05 mg. ammonium nitrogen, then, in all cases examined (twenty), with but one exception, a perfectly colorless solution resulted on heating them with 1.0 per cent aqueous ninhydrin solution (Table II). The one exception is ammonium sodium hydrogen phosphate, which gives a very faint trace of violet color under those conditions and the probable cause of this exception will be discussed later.

In the majority of cases we have been able to identify the blue or violet coloration as due, at any rate in part, to the ammonium salt of diketohydrindylidene-diketohydrindamine. We used the three tests mentioned on page 322, but in no case was sufficient color present to be determined quantitatively by the Harding-MacLean colorimetric method.[13] The discrepancies of previous observers can thus be accounted for; in concentration of about 1 per cent the ammonium salts of the weak organic acids give an unmistakable ninhydrin reaction, which is not the case when the concentration is extremely low (1.0 cc. = 0.05 mg. ammonium nitrogen).

In view of the results it became of interest and importance to determine whether ammonium salts in very dilute solution gave a ninhydrin reaction in the presence of pyridine. This modification of the ninhydrin reaction discovered by Harding and MacLean[13] results in a quantitative decomposition of the α-amino nitrogen of amino-acids. How far, if at all, does it affect the decomposition of ammonium nitrogen when in similar concentration? The method of investigation was as follows:

Standard solutions of different ammonium salts were prepared of such a strength that 1 cc. of solution contained 0.05 mg. of ammonium nitrogen, thus being equivalent in strength to the standard alanine.

1 cc. of the ammonium salt solution together with 1 cc. of 10 per cent aqueous pyridine and 1 cc. of 1 per cent ninhydrin were heated for a period of 20 minutes in a rapidly boiling water bath. A strong blue color developed. The contents of the test-tube were cooled, diluted to 50 cc., and the amount of color was measured against a standard alanine color simultaneously prepared.

[13] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1915, xx, 217.

For reasons given in a previous paper[14] the amount of decomposition of alanine, etc., is assumed to be quantitative. It will be seen by a glance at Table II that the decomposition of ammonium nitrogen under these conditions is constant and independent of the acid attacked, the two columns representing duplicate determinations.

TABLE II.

Ammonium salt.	Without pyridine.	With pyridine. Nitrogen decomposed.	
		mg.	*mg.*
Chloride	No color.	0.019	0.020
Nitrate	" "	0.018	0.018
Sulfate	" "	0.017	0.016
Sodium hydrogen phosphate.	Very pale violet color.	0.016	0.015
Hydrogen phosphate	No color.	0.019	0.019
Carbonate	" "	0.018	0.018
Formate	" "	0.019	0.019
Acetate	" "	0.020	0.020
Oxalate	" "	0.017	0.018
Succinate	" "	0.020	0.019
Glycollate	" "	0.019	0.019
Lactate	" "	0.019	0.019
Malate	" "	0.020	0.019
Tartrate	" "	0.018	0.019
Citrate	" "	0.015	0.015
β-Oxybutyrate	" "	0.020	0.020
Benzoate	" "	0.020	0.022
Cinnamate	" "	0.020	0.020
Opianate	" "	0.018	0.018
Thiocyanate	" "	0.019	0.019

This decomposition of ammonium nitrogen is constant at about 0.018 mg. in 0.05 mg., or about 36 per cent, and the identification of the coloring matter as the ammonium salt of diketohydrindylidene-diketohydrindamine is readily effected by the use of the three afore mentioned tests.

We next investigated the effect of varying amounts of ninhydrin and pyridine upon the reaction under conditions which parallel those in our studies on the decomposition of alanine. This is shown in Table III.

[14] Harding and MacLean, *J. Biol. Chem.*, 1916, xxiv, 503.

TABLE III.

Ammonium salt. 1 cc. = 0.05 mg. N.	Pyridine concentration (per cent).				Ninhydrin concentration (per cent).		N decomposed.
	5	10	15	25	1	2	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*mg.*
Chloride	1				1		0.010
"		1			1		0.017
"			1		1		0.019
"				1	1		0.020
"		1				1	0.024
"		1			2		0.025
Sulfate	1				1		0.010
"		1			1		0.018
"			1		1		0.019
"				1	1		0.020
"		1				1	0.025
"		1			2		0.026
Nitrate		1			2		0.025
Acetate		1			2		0.025
Benzoate		1			2		0.026

1 cc. of the ammonium salt was heated with the amount of pyridine and ninhydrin stated, in a boiling water bath for a period of 20 minutes, and the amount of ammonium nitrogen decomposed was found in the usual way. It will be seen that concentrations of pyridine higher than 10 per cent have very little influence, though there is to be noted a small but distinct rise. In this respect the reaction of an ammonium salt differs from that of alanine. An increase in the amount of ninhydrin, even if the ninhydrin be used in the same concentration, causes a marked increase in coloring matter. With alanine, no such increase is noted.

We have not made any extensive studies on the influence of time on this reaction. At the end of 20 minutes, however, the reaction is mainly complete. Thus an isolated observation on ammonium β-hydroxybutyrate showed that an hour's heating at 100°C. only increased the decomposition of the nitrogen from 0.020 to 0.027 mg.

In summing up the experimental results, it will be seen that all ammonium salts under the right conditions are capable of giving

the ninhydrin reaction. Those conditions appear to be a faint alkalinity of solution, brought about either by the ammonium salt itself or by the presence of pyridine. The coloring matter formed agrees in properties with the ammonium salt of diketohydrindylidene-diketohydrindamine prepared either synthetically or by the interaction of alanine and triketohydrindene hydrate, and there thus remains the task of showing how ammonium salts in general can interact with Ruhemann's reagent and give an end-product identical with that produced from amino-acids. The simplest and most obvious explanation, that amino-acids pass into ammonium salts, is inadequate, for if so, the decompositions should either parallel one another or ammonium salts should be more reactive than amino-acids, statements which are not in agreement with experimental facts. Of the two, amino-acids are by far the more reactive. They react in much more dilute solutions than ammonium salts and, in the presence of pyridine, are nearly three times as strong in their action.

The Mechanism of the Ninhydrin Reaction.

Ruhemann's[7] view of the interaction of amino-acids and triketohydrindene hydrate involves the oxidation of the amino-acid to carbon dioxide and an aldehyde possessing a carbon atom less than the amino-acid with the simultaneous reduction of the triketone to hydrindantin and the condensation of the hydrindantin with the ammonia liberated by the oxidation of the amino-acid, forming the blue colored ammonium salt of diketohydrindylidene-diketohydrindamine. Such a mechanism, while satisfactory for amino-acids, does not explain the positive ninhydrin reaction with ammonium salts, especially of inorganic acids. Adopting Ruhemann's view of the constitution of the blue coloring matter, we have sought to determine the mechanism whereby the amino-acid acts as a reducing agent and to see whether any parallel reaction was possible with an ammonium salt.

In this respect we have utilized the theory of Dakin and Dudley,[15] who have postulated that amino-acids undergo either a dissociation or a decomposition into ammonia and the corresponding glyoxal.

[15] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913. xv 127.

$$RCHNH_2.COOH \rightarrow RCO.CHO + NH_3$$

Their later work has inclined them to the belief that the reaction expressed is not a true chemical dissociation. In the case of alanine this would lead to the formation of methyl glyoxal and ammonia.

$$CH_3.CHNH_2.COOH \rightarrow CH_3.CO.CHO + NH_3$$

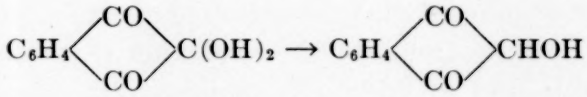
The glyoxals as a class are distinguished by being powerful reducing agents; they reduce ammoniacal silver oxide and Fehling's solution even in the cold. Thus it would be expected that a reduction of triketohydrindene hydrate would take place, giving rise to a substituted glyoxylic acid,

$$CH_3.CO.CHO + O = CH_3.CO.COOH$$

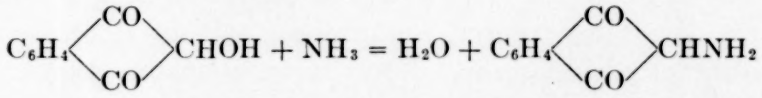
and that this would decompose into carbon dioxide and an aldehyde.

$$CH_3.CO.COOH = CH_3.CHO + CO_2$$

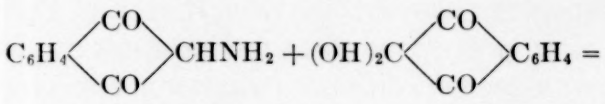
The triketohydrindene hydrate would be reduced to 1, 3-diketohydrindol

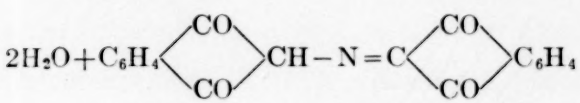


which on condensation with the ammonia from the amino-acid would give 1, 3-diketohydrindamine.



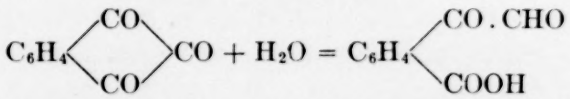
This compound readily condenses with aldehydes and ketones, is readily oxidized to a deep blue coloring matter,[3,7] and consequently would be expected to condense with a molecule of triketohydrindene hydrate to give diketohydrindylidene-diketohydrindamine, the ammonium salt of which is the required blue coloration.





Such a scheme accounts satisfactorily for the end-products of the reaction. It differs from that outlined by Ruhemann by not regarding hydrindantin as an intermediate product. The direct action of ammonia upon hydrindantin to produce diketohydrindylidene-diketohydrindamine would be expressed much better by a series of simple reactions involving the hydrolysis of hydrindantin to diketohydrindol and triketohydrindene hydrate. Moreover, we believe that we possess a certain amount of evidence against the view, for the presence of hydrindantin in large amounts reduces the amount of diketohydrindylidene-diketohydrindamine formed from an ammonium salt (page 333).

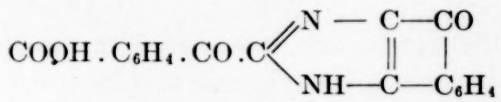
Again, as a working hypothesis, the intermediate formation of a glyoxal is attractive to us because it enables us to explain the ninhydrin reaction with ammonium salts. It is to be noticed that the ninhydrin reaction in the presence of pyridine is the same for all ammonium salts, being entirely independent of the acid attached; without pyridine it is most evident with the ammonium salts of the weak organic acids; *i.e.*, those salts which are the most readily hydrolyzed into their basic and acidic components, and with those salts which are faintly alkaline in reaction; *e.g.*, sodium ammonium hydrogen phosphate. Any reduction of the triketohydrindene hydrate under these circumstances must come from itself and the first action of the pyridine, or the weakly alkaline or dissociated salt must be the action of the hydroxyl ions upon the triketone. Ruhemann has shown conclusively that the first action of alkalies upon triketohydrindene hydrate is the formation of phenylglyoxal-*o*-carboxylic acid.



In other words, under the influence of weak alkalies the triketone itself can furnish the necessary glyoxal and the reaction would then proceed in the manner previously indicated. The formation of the yellow colored glyoxal can readily be observed on adding a few drops of 10 per cent aqueous pyridine to the triketone.

A necessary consequence of the hypothesis is that ammonia itself should give the ninhydrin reaction. Ruhemann investigated the action of ammonia upon the triketone and found

that he obtained a deep brownish red solution, from which by acidification he obtained a compound he regarded as 2-*o*-carboxybenzoylindoglyoxaline,



The formation of the phenylglyoxal-*o*-carboxylic acid is evident, and this undergoes condensation with ammonia and a second molecule of triketohydrindene hydrate. We have found, however, that in very dilute solution the reaction with ammonia follows the reaction with ammonium salts. In a concentration of 1 cc. = 0.05 mg. ammonia nitrogen no reaction is obtained on heating 1 cc. with 1 cc. of 1 per cent ninhydrin for 20 minutes in a boiling water bath. In the presence of 1 cc. of 10 per cent aqueous pyridine, the ammonium salt of diketohydrindylidene-diketohydrindamine is formed and the decomposition of the ammonia nitrogen is 0.013 mg., a figure not far removed from the constant given by ammonium salts.

It is also easy to see why β- and γ-amino-acids have been found to give a faint ninhydrin reaction, and why acids with an amino group attached to a benzene ring fail to give the test, though we should expect that even here those aromatic amino-acids of which the amino group is readily hydrolyzed, would give a feeble positive reaction.

The Action of Reducing Agents on the Ninhydrin Reaction with Ammonium Salts.

It is evident that reducing reagents which assist the production of diketohydrindol from triketohydrindene hydrate should increase the amount of coloring matter formed with ammonium salts. And this in general we have found to be the case. We have examined a number of organic reducing agents which from their known reactions might be expected not to interfere with the condensations which take place, and measured their influence on the amount of coloring matter formed.

1 cc. of the standard solution of ammonium salt, 1 cc. of 10 per cent aqueous pyridine, 1 cc. of 2 per cent ninhydrin solution, and 1 cc. of the

reducing agent of the strength shown in Table IV were heated together in a boiling water bath for 20 minutes and the amount of nitrogen decomposed was estimated in the usual way. Control experiments on the reducing agent and ninhydrin in presence of pyridine gave negative results.

TABLE IV.

Ammonium salt.	Reducing agent.	Concentration of reducing agent.	N decomposed.
		per cent	*mg.*
Chloride	Ethylene glycol.	10	0.025
"	" "	100	0.035
"	Glycerol.	10	0.027
"	"	100	0.032
"	*p*-Hydroxybenzaldehyde.	1	0.029
"	*p*-Hydroxybenzaldehyde.	3	0.032
"	Glucose.	0.5	0.025
"	"	5.0	0.032
Nitrate	"	0.5	0.027
"	"	5.0	0.031
Sulfate	"	0.5	0.026
"	"	5.0	0.032
Acetate	"	0.5	0.026
"	"	5.0	0.032
Benzoate	"	0.5	0.026
"	"	5.0	0.032
Constant for ammonium salt	1 cc. water.		0.025

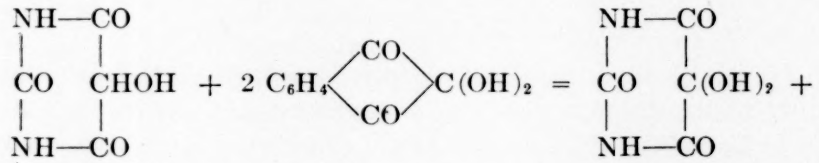
It will be seen that reducing agents have a distinct influence on the decomposition of ammonium nitrogen, though it is not so great as might have been expected, and in all cases a high concentration is necessary before the effect is shown. Thus a 0.5 per cent solution of glucose is without any influence and a 5 per cent solution only raises the decomposition from 50 to 64 per cent.

The results with ethylene glycol and glycerol are of interest, as they have a distinct bearing on the claims of Halle, Loewenstein, and Přibram[16] that these substances themselves give a ninhydrin reaction. They appear to act as very weak reducing

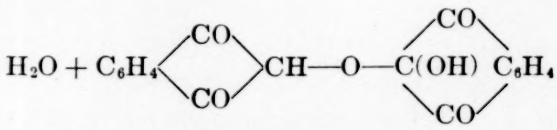
[16] Halle, W., Loewenstein, E., and Přibram, E., *Biochem. Z.*, 1913, lv, 357.

agents only, but this will be discussed more fully in another communication. The poor results obtained go to show that the reducing agent which takes part in the ninhydrin reaction must be either a very specific one or exceptionally powerful.

Of all the reducing agents we have examined dialuric acid is by far the most interesting. This acid was shown by Ruhemann[17] to give excellent yields of hydrindantin when heated with triketohydrindene hydrate, and we have repeated the observation several times in this laboratory; indeed, it is not necessary to heat the solutions, for in the concentrations at which we were working the hydrindantin rapidly crystallized out at the ordinary temperature.



Dialuric acid. Alloxan.



Hydrindantin.

A cold saturated solution of dialuric acid was prepared by dissolving the acid in hot water, cooling, and allowing the excess of acid to crystallize out over night (A). A second solution was prepared by dissolving dialuric acid in hot water, of such a strength that 1 cc. was capable of converting the whole of the triketohydrindene hydrate present in 1 cc. of a 2 per cent solution into hydrindantin according to the above equation (B). Such a solution of dialuric acid is supersaturated at ordinary temperatures, but as the dialuric acid does not crystallize out immediately, no difficulty is experienced in its use. Table V shows the effect of dialuric acid as a reducing agent on the ninhydrin reaction with ammonium salts, the experimental details being the same as t' ose described on pages 330–331, and the figures representing mg. of ammonium nitrogen decomposed.

[17] Ruhemann, *J. Chem. Soc.*, 1911, xcix, 1310.

TABLE V.

Ammonium salt.	+ 1 cc. of water.	Dialuric acid.	
		(A)	(B)
	mg.	*mg.*	*mg.*
Chloride	0.025	0.045	0.031
Nitrate	0.025	0.044	0.031
Sulfate	0.026	0.043	0.031
Acetate	0.025	0.045	0.032
Benzoate	0.026	0.042	0.031

The striking influence of small amounts of dialuric acid is at once apparent. The cold saturated solution of the acid raises the decomposition of the ammonium nitrogen to nearly 90 per cent. The effect of the second solution of dialuric acid is not so marked. Here the solution contains a large excess of hydrindantin and its effect is not so great as that of smaller amounts; it is this fact that has induced in us the belief that hydrindantin probably does not form an intermediate step in the ninhydrin reaction. Moreover, the colors produced in the second series are of a very red shade, making the matching with the standard very difficult. In order to see if these red shades of color were due to the presence of alloxan or any derivative of it, or murexide, the hydrindantin formed by the interaction of the dialuric acid and the triketohydrindene hydrate was filtered off, washed on the filter with a little cold water, suspended in 2 cc. of distilled water, and a quantitative ninhydrin reaction was carried out with it. The solution obtained was extremely red in color and was found to contain only 0.013 mg. of decomposed nitrogen. A second experiment with pure crystallized hydrindantin gave an almost exactly similar result. The production of the reddish yellow colorations, then, is brought about by the excess of hydrindantin which does not decompose readily to give the ammonium salt of diketohydrindylidene-diketohydrindamine by the action of ammonia.

As the action of the cold saturated solution of dialuric acid had raised the decomposition of the ammonium salt to nearly 90 per cent, it became of interest and perhaps of practical importance to see if conditions could not be found under which the decomposition would become quantitative. Consequently a series of ex-

periments were performed by heating 1 cc. of standard ammonium chloride solution with 1 cc. of 10 per cent aqueous pyridine and 1 cc. of 2 per cent ninhydrin solution, with 1 cc. of dialuric acid solution of various strengths for varying periods of time in a rapidly boiling water bath. The results are shown in Table VI.

TABLE VI.

Dialuric acid per 1 cc.	Time of heating.	N decomposed.
mg.	*min.*	*mg.*
0.000	20	0.025
0.514	20	0.032
1.028	20	0.043
1.542	20	0.045
2.056	20	0.046
2.570	20	0.045
3.084	20	0.044
4.112	20	0.042
5.140	20	0.039
2.056	10	0.029
2.056	15	0.037
2.056	20	0.045
2.056	30	0.045
2.056	40	0.044

The decomposition of ammonium nitrogen by triketohydrindene hydrate in the presence of pyridine never becomes complete. The optimum decomposition occurs in the presence of a solution of dialuric acid containing 2.056 mg. per cc. when the time of heating is 20 minutes. These conditions are practically those of Table V with the cold saturated solution of dialuric acid (A). Increase in time of heating does not affect the amount of coloring matter, but with larger amounts of dialuric acid the color gradually decreases and also acquires a strong reddish shade which renders its comparison with the alanine standard very difficult and uncertain.

SUMMARY.

1. Methods of detection of the ammonium salt of diketohydrindylidene-diketohydrindamine are given.

2. In a concentration of 1 per cent the ammonium salts of weak acids react positively with ninhydrin.

3. In very high concentration the ammonium salts of the strong mineral acids give a positive ninhydrin reaction.

4. In a concentration of 1 cc. = 0.05 mg. of ammonium nitrogen, no reaction is obtained.

5. In the presence of pyridine and in a concentration of 1 cc. = 0.05 mg. of nitrogen, all ammonium salts react positively with triketohydrindene hydrate. The amount of decomposition of the ammonium nitrogen was found to be independent of the acid radicle attached and possessed a constant value of 0.018 mg. nitrogen; the blue coloration is due to the ammonium salt of diketohydrindylidene-diketohydrindamine.

6. A mechanism of the ninhydrin reaction with amino-acids is suggested, depending on the decomposition of the amino-acid into ammonia and the corresponding glyoxal.

7. The formation of phenylglyoxal-*o*-carboxylic acid by the hydrolysis of triketohydrindene hydrate is supposed to be the first stage in the mechanism of the ninhydrin reaction with ammonium salts.

8. Reducing agents increase the ninhydrin reaction with ammonium salts.

THE NINHYDRIN REACTION WITH AMINES AND AMIDES.

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In the preceding paper Harding and Warneford[1] have examined and discussed the interaction of amino-acids and ammonium salts with triketohydrindene hydrate (ninhydrin). They showed that the ninhydrin reaction with amino-acids could be explained by adopting the hypothesis of Dakin and Dudley[2] which supposes the decomposition of the amino-acid into ammonia and the corresponding glyoxal,

$$RCHNH_2 . COOH \rightarrow RCO . CHO + NH_3$$

the glyoxal acting as the reducing agent. They showed that the ninhydrin reaction with ammonium salts, first pointed out as a general reaction by Neuberg,[3] only took place in the presence of hydroxyl ions, and that these had been observed by Ruhemann[4] to hydrolyze triketohydrindene hydrate to phenylglyoxal-*o*-carboxylic acid.[5]

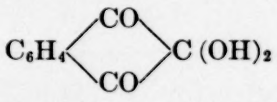
[1] Harding, V. J., and Warneford, F. H. S., *J. Biol. Chem.*, 1916, xxv, 319.

[2] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 127.

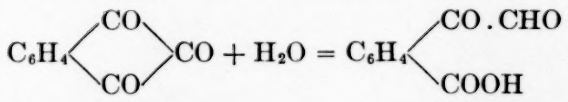
[3] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.

[4] Ruhemann, S., *J. Chem. Soc.* 1910, xcvii, 2026.

[5] In this and the preceding paper triketohydrindene hydrate has sometimes been written as the triketone for the sake of simplicity in explaining the decompositions. Its true formula, as pointed out by Ruhemann is,



and it will be found that we have used this where the reactions could be more clearly shown by its use.



In this way the requisite glyoxal was produced and the two series of facts were brought into agreement.

There still remains, however, the second criticism brought against the ninhydrin reaction by Neuberg,[3] who noticed that a large number of organic bases give a positive test with triketohydrindene hydrate. This is in contradiction to the statements of Abderhalden and Schmidt.[6]

In our experimental method we have followed the same procedure as that adopted in the previous paper. The bases examined were tested in varying concentration, with and without the presence of pyridine, and the general results followed those obtained with ammonium salts.

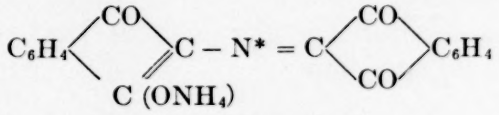
TABLE I.

Base.	1 per cent solution.	Solution: 1 cc. = 0.05 mg. N.		
		1 per cent ninhydrin.	N decomposed in	
			1 per cent ninhydrin and pyridine.	2 per cent ninhydrin and pyridine.
			mg.	*mg.*
Methylamine	Strong reaction.	No reaction.	0.041	0.045
Ethylamine	" "	" "	0.037	0.044
Allylamine	" "	" "	0.021	0.022
Pentamethylenediamine	Reddish shades.	" "	+	+
Glucosamine	Strong reaction.	" "	0.043	0.046
Ethyl glycocoll	" "	" "	0.036	0.037
Phenocoll	" "	" "	0.021	0.021
Isopropylamine	No "	" "	No reaction.	
Aniline	" "	" "	" "	
Diethylamine	" "	" "	" "	
Diisopropylamine	" "	" "	" "	
ω-Aminoacetophenone	" "	" "	" "	

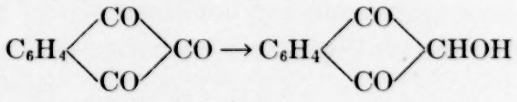
It is seen from an inspection of Table I that those bases which react with triketohydrindene hydrate do so in relatively high con-

[6] Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, 1911, lxxii, 37.

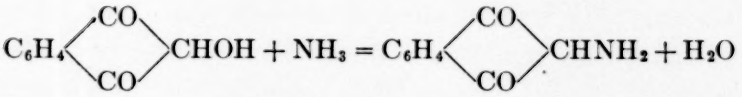
centration only. When in very dilute solution (1 cc. = 0.05 mg. amino nitrogen), no reaction is obtained. In the presence of pyridine, however, those bases which react in the higher concentration are found to react even in the very dilute solution. In this respect the ninhydrin reaction with amines follows that with ammonium salts. It is to be noted that if a base does not react with triketohydrindene hydrate alone when in the higher concentration neither does it do so when in the lower concentration and in the presence of pyridine. Furthermore, the amount of coloring matter (ammonium salt of diketohydrindylidene-diketohydrindamine) formed by the reactive bases in dilute solution in presence of pyridine is not constant, as it is in the case of the ammonium salts. The amount of color formed is distinctly and clearly dependent on the constitution of the amine from which it is formed, a point which we will discuss later. The identity of the coloring matter was proved by the three tests mentioned in the previous paper.[1] The constitution given to this compound,



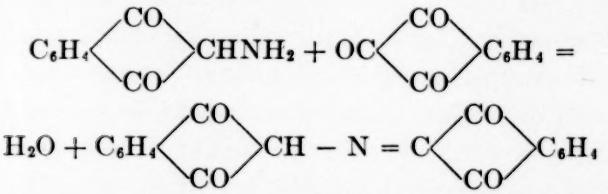
and its formation by the interaction of certain bases with triketohydrindene hydrate, mean a complete decomposition of the amines, in order to supply the nitrogen atom marked with the asterisk. The salt need not necessarily be an ammonium salt, as a salt with an organic base would fit the facts equally well, but the substitution of any other group for the nitrogen atom marked with the asterisk, or an alteration in the linkages, would cause a marked alteration in the absorption spectrum. In explaining the mechanism of the ninhydrin reaction with amino-acids, the nitrogen atom was supplied by the decomposition of the acid into the corresponding glyoxal and ammonia, the former causing a reduction of the triketone to 1, 3-diketohydrindol,



which condensed with loss of water with the ammonia.



A second molecule of triketohydrindene hydrate then condensed with the 1, 3-diketohydrindamine



to give diketohydrindylidene-diketohydrindamine. In the reaction with the ammonium salts the glyoxal was supplied by the hydrolysis of part of the triketone, the ammonia necessary to supply the nitrogen atom coming from the ammonium salt. The production of ammonia, however, from such organic amines as methylamine and ethylamine is a matter of difficulty. Such bases do not hydrolyze readily into the corresponding alcohol and ammonia. Any dissociation theory, such as that of methylamine into ammonia and methylene,[7]

$$CH_3NH_2 \rightleftharpoons CH_2: + NH_3$$

has not sufficient facts in its favor to warrant its assumption. Moreover, such a dissociation theory would fail to explain the negative reaction with isopropylamine.

As the ninhydrin reaction with amines is clearly dependent on the constitution of the base, it becomes important to see if some insight into its mechanism cannot be gained by a classification of the bases, according to their action towards triketohydrindene hydrate and to their chemical constitution. This has been done and the results are given in the following tables.

In tabulating our own results we have always taken into consideration the concentration of the base, and a negative reaction given by us means that the base gives no blue coloring matter when 1 cc. of a 1 per cent solution is heated with 1 cc. of 1 per cent ninhydrin solution in a boiling water bath for a period of 20 minutes.

[7] Compare Hurtley, W. H., and Wooton, W. O., *J. Chem. Soc.*, 1911, xcix, 291.

TABLE II.

Type: RCH_2NH_2.

Base.	Observer.	Reaction.	Remarks.
Methylamine	Neuberg.	+	
	Harding and MacLean.	+	
Ethylamine	Neuberg.	+	
	Harding and MacLean.	+	
Isobutylamine	Neuberg.	+	Somewhat reddish.
Isoamylamine	"	+	
Hexylamine	"	+	Reddish.
Allylamine	"	−	Yellowish red.
	Harding and MacLean.	+	Reaction readily identified.
Benzylamine	Neuberg.	+	Reddish.
Phenylethylamine	"	+	
p-Hydroxyphenylethylamine	"	+	
β-Iminazolethylamine	"	+	
β-Indolethylamine	"	+	
Ethylenediamine	"	+	
Tetramethylenediamine	"	+	
Pentamethylenediamine	"	+	Reddish.
	Harding and MacLean.		
Aminoethylalcohol	Neuberg.	+	
Aminoacetal	"	+	
Aminoacetaldehyde	"	+	
Ethyl glycocoll	Ruhemann.	−	
	Harding and MacLean.	+	
Phenocoll	" " "	+	
ω-Aminoacetophenone	Neuberg.	+	
Taurine	"	+	In presence of sodium acetate.

The results given by this type of amine are clear and unequivocal. The only two exceptions previously known, *i.e.*, allylamine and ethyl glycocoll, on examination are shown to be errors. The finding with ethyl glycocoll ($H_2NCH_2CO_2C_2H_5$) and phenocoll ($C_2H_5OC_6H_4NHCOCH_2NH_2$) is of great interest as it finally dis-

poses of the claim of Ruhemann[8] and Abderhalden and Schmidt[6] that it is necessary for both the carboxyl and amino group of an amino-acid to be unsubstituted in order to obtain a positive result. The carboxyl group may be substituted, but substitution on the amino group certainly prevents the reaction (hippuric acid).

TABLE III.

Type: R_2CHNH_2.

Base.	Observer.	Reaction.	Remarks.
Isopropylamine.....	Harding and MacLean.	−	
Camphylamine.....	Neuberg.	−	Reddish.
Glucosamine.....	Abderhalden and Schmidt.	−	
	Neuberg.	+	
	Harding and MacLean.	+	Strong reaction.

The very small number of bases of this type which have been examined makes it difficult to draw definite conclusions. It would appear, however, that simple bases of this type do not react with triketohydrindene hydrate (isopropylamine and camphylamine). In the case of glucosamine, where we found an undoubted reaction, we have a negative aldehyde group on the carbon atom adjacent to the amino group, a fact which is well known to increase the reactivity of the neighboring groups, and the explanation of the reactivity of glucosamine may be sought in this direction. Moreover, it must not be forgotten that the amino-acids which are obtained by the hydrolysis of proteins and which show the greatest reactivity, belong to this group (except glycine). A number of di- and tri-peptides, quoted by Abderhalden and Schmidt as giving a positive reaction, are also to be classed in this category, so that the position occupied by glucosamine is not an isolated one. As a consequence we are brought to the conclusion that the grouping R_2CHNH_2, when one of the radicles is negative in character, reacts with ninhydrin, otherwise it is unreactive.

With the exception of the two tertiary amino-acids examined by Ruhemann, bases of this type do not give any reaction with ninhydrin. The two exceptions mentioned probably give a posi-

[8] Ruhemann, *J. Chem. Soc.*, 1911, xcix, 798.

TABLE IV.

Type: R_3CNH_2.

Base.	Observer.	Reaction.
α-Aminoisobutyric acid	Ruhemann.	+
α-Aminoethylbutyric acid	"	+
β-Aminocrotonic ester	Neuberg.	−
Adenine	"	−
Guanine	Abderhalden and Schmidt.	−
	Harding and MacLean.	−
Aniline	Neuberg.	−
	Harding and MacLean.	−
Benzidine	" " "	−
p-Aminoacetophenone	" " "	−

tive reaction on account of the ease with which tertiary amino-acids lose ammonia to form unsaturated acids. Indeed, Ruhemann[8] himself makes the observation that these two amino-acids required boiling with the reagent in order to give the reaction, whereas the other amino-acids only required warming.

TABLE V.

Types: R_2NH *and* R_3N.

Base.	Observer.	Reaction.
Diethylamine	Neuberg.	−
	Harding and MacLean.	−
Diisopropylamine	" " "	−
Diisobutylamine	" " "	−
Piperidine	Neuberg.	−
	Harding and MacLean.	−
Adrenaline	Neuberg.	+*
	Harding and MacLean.	+*
Indole	Neuberg.	−
Skatole	Harding and MacLean.	−
Trimethylamine	" " "	−
Triethylamine	Neuberg.	−
	Harding and MacLean.	−
Pyridine	Neuberg.	−
	Harding and MacLean.	−
Quinoline	Neuberg.	−
	Harding and MacLean.	−
Isoquinoline	Neuberg.	−

* Red shades.

With the exception of adrenaline secondary and tertiary bases are unreactive towards triketohydrindene hydrate. The instability of solutions of adrenaline is well known and this exception can well be explained in this way.

It is evident, then, that there are only two general classes of organic bases which give the ninhydrin reaction, RCH_2NH_2 and R_2CHNH_2 where one radicle is strongly negative in character.

In attempting to explain the ninhydrin reaction with these classes of organic bases, two facts must be borne in mind: (1) The reaction does not take place when the amine is in very dilute solution, unless in the presence of pyridine; *i.e.*, it parallels the reaction with ammonium salts. (2) The extent of the reaction depends on the constitution of the amine, the simpler amines being the more reactive unless a negative group is present (Table I).

In the previous paper we have shown how the analogy between triketohydrindene hydrate and alloxan enabled Ruhemann to arrive at the constitution of the blue color produced by the interaction of triketohydrindene hydrate and amino-acids. The analogy, however, between the formation of murexide from alloxan and its derivatives and the formation of the ammonium salt of diketohydrindylidene-diketohydrindamine in the ninhydrin reaction is much closer than has previously been recognized. In Tables VI and VII we have collected the results of the different investigators in the two fields. In Table VI we have collected the work of Ruhemann. The analogies, which were first drawn by him, are very clear and striking and extend not only to the methods of preparation of the compounds but to many of their physical and chemical properties. This is particularly striking in the case of the formation of chromo salts. It also shows the formation of 7-ethyluramil by the action of ethylamine on alloxantin, an observation which is as yet unknown in the hydrindene series. Table VII shows the relationships in the formation of murexide and the ammonium salt of diketohydrindylidene-diketohydrindamine. It shows that murexide can be formed by the action of ammonia, alanine (amino-acids), or ethylamine upon alloxan or alloxantin. If the analogy is a true one it is not surprising, then, to find that ammonia and ammonium salts, and organic bases of the type of ethylamine, in addition to amino-acids react with triketohydrindene hydrate to give the ninhydrin reaction.

TABLE VI.

The Relation of Alloxan and Triketohydrindene Hydrate.

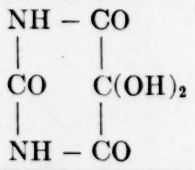
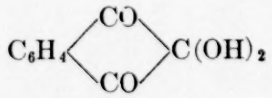
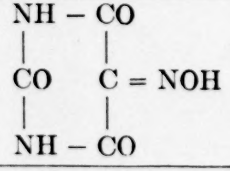
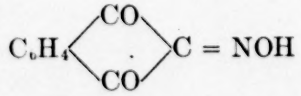
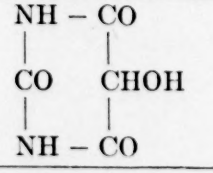
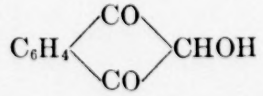
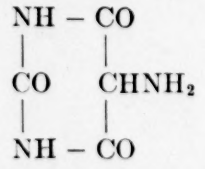
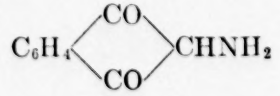
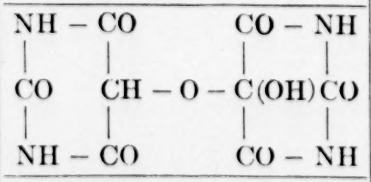
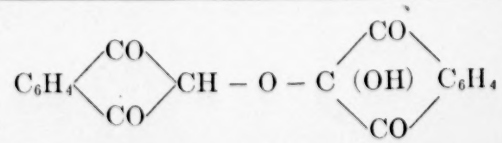
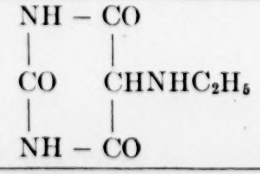
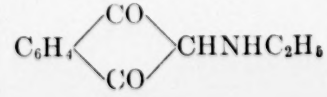
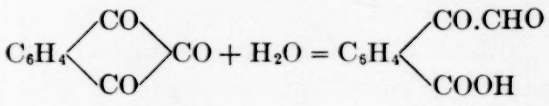
Alloxan and derivatives.			Triketohydrindene hydrate and derivatives.		
Compound.	Preparation.	Formula.	Compound.	Preparation.	Formula.
Alloxan.			Triketohydrindene hydrate.		
Violuric acid.	Hydroxylamine on alloxan. Nitrous acid on barbituric acid.		Oximo 1, 3-diketohydrindene.	Hydroxylamine on triketohydrindene hydrate. Nitrous acid on 1, 3-diketohydrindene.	
Dialuric acid.	Reduction of alloxan by hydriodic acid.		1, 3-Diketohydrindol.	Reduction of triketohydrindene hydrate by sodium amalgam.	
Uramil.	Reduction of violuric acid. Action of ammonium chloride on hydrindantin.		1, 3-Diketohydrindamine.	Reduction of oximo 1, 3-diketohydrindene.	
Alloxantin.	Reduction of alloxan by H_2S.		Hydrindantin.	Reduction of triketohydrindene hydrate by H_2S.	
7-Ethyluramil.	Action of ethylamine on alloxantin.		1,3-Diketoethylhydrindamine.	Unknown.	

TABLE VII.

The Relationship of Murexide and the Ammonium Salt of Diketohydrindylidene-diketohydrindamine.

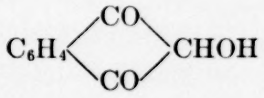
Murexide.		Ammonium salt of diketohydrindylidene-diketohydrindamine.	
Preparation from	By the action of	Preparation from	By the action of
Alloxan.	Ammonia in alcoholic solution. Ethylamine. Amino-acids.	Triketohydrindene hydrate.	Ammonia in very dilute solution in presence of pyridine. Ethylamine. Amino-acids.
Alloxantin.	Ammonium acetate and carbonate. Ethylamine. Amino-acids.	Hydrindantin.	Ammonium acetate and carbonate. Amino-acids.
Uramil.	I_2 or HgO. Alloxan in presence of ammonia.	1,3-Diketohydrindamine.	Air.
7-Ethyluramil.	Alloxan.		

The papers of Piloty and Finckh,[9] Slimmer and Stieglitz,[10] Möhlau,[11] and Möhlau and Litter[12] supply us with the clue to the ninhydrin reaction with amines. The similarity of the reaction with amines to that with ammonium salts in requiring the presence of large amounts of base or the presence of pyridine, again suggests that the production of phenylglyoxal-*o*-carboxylic acid represents the first stage in the mechanism

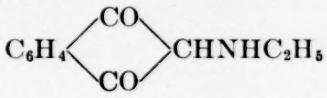


[9] Piloty, O., and Finckh, K., *Ann. Chem.*, 1904, cccxxxiii, 22.
[10] Slimmer, M., and Stieglitz, J., *Am. Chem. J.*, 1904, xxxi, 661.
[11] Möhlau, R., *Ber. chem. Ges.*, 1904, xxxvii, 2686.
[12] Möhlau, R., and Litter, H., *J. prakt. Chem.*, 1906, lxxiii, 449.

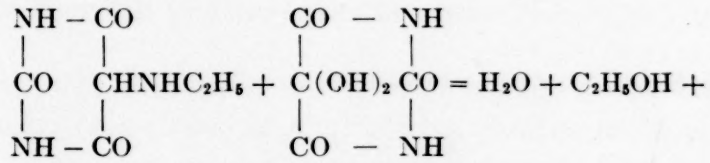
and that this acts as a reducing agent giving 1, 3-diketohydrindol or hydrindantin.

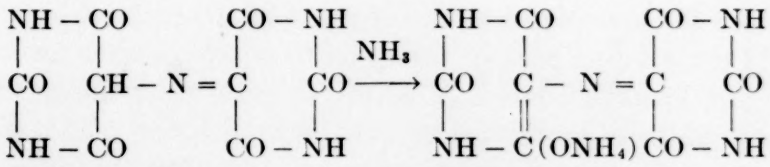


The action of ethylamine on alloxantin, as observed by Piloty and Finckh,[9] Slimmer and Stieglitz,[10] and Möhlau and Litter,[12] gives 7-ethyluramil, and there is no reason to suppose that the action of ethylamine on 1, 3-diketohydrindol or on hydrindantin would not produce 1, 3-diketoethylhydrindamine.



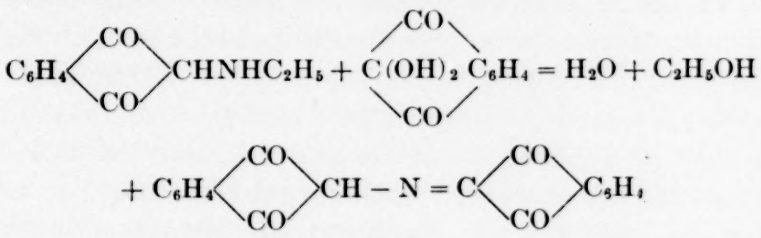
Just as 7-ethyluramil and alloxan in the presence of ammonia[12] give the ammonium salt of purpuric acid (murexide) and ethyl alcohol





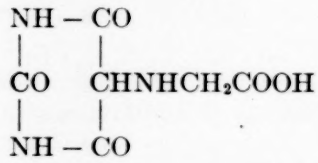
Purpuric acid. Murexide.

so it would be expected that 1, 3-diketoethylhydrindamine and triketohydrindene hydrate would undergo a similar condensation to give diketohydrindylidene-diketohydrindamine



which would give a blue salt with either an excess of ethylamine or pyridine. It is this latter reaction, *i.e.*, the removal of ethyl alcohol by condensation, which probably represents the constitutive part of the ninhydrin reaction with bases, as it is well known that condensations depending on the removal of an alcohol become more and more difficult as the molecular weight of the alcohol increases.

It must not be forgotten, however, that amino-acids themselves may also react in the presence of pyridine according to this mechanism, as well as by the mechanism expressed in the previous paper. Indeed, Piloty and Finckh claim to have isolated from the interaction of alloxan and glycine a compound possessing the constitution of uramiloacetic acid,



though Hurtley and Wooton[7] take an entirely different view of the reaction.

This view of the mechanism of the ninhydrin reaction with amines also is not entirely satisfactory, as it does not explain why bases of the type of isopropylamine are unreactive, and there is still room for investigation on this part of the ninhydrin reaction. Lack of material at present forces us to postpone such an inquiry.

We are, however, strongly of the opinion that the production of ammonia from amino-acids and the production of the nitrogen atom marked with an asterisk (page 339) from amines is not brought about by hydrolysis, and are confirmed in this belief by the behavior of amides towards ninhydrin. This class of compounds are generally readily hydrolyzed to give ammonia, and yet they give only the faintest of ninhydrin reactions in the presence of pyridine. Alone, in a 1 per cent solution, only two of them showed any reaction, urea and allantoin, and these, though definite, are weak. The results are tabulated in Table VIII. Substituted amides, such as hippuric acid and glycocholic acid, cyclic imides, such as the various cyclic ureides, and the imino compounds, such as guanidine, creatine, and creatinine, do not give any reaction, either with or without the presence of pyridine.

The non-reactivity of the guanidine grouping is important from our standpoint as it means that the quantitative ninhydrin reaction worked out by us is confined to the α-amino group of arginine. The slight reactivity of pentamethylenediamine also leads us to the conclusion that the amino group in lysine will not introduce any serious error into our method.

TABLE VIII.

Amide.	1 per cent solution, without pyridine.	1 cc. amide solution (0.05 mg. N). 1 cc. pyridine (10 per cent). 1 cc. ninhydrin (1 per cent).
Formamide*.....	No reaction.	Very faint reaction.
Acetamide.....	" "	" " "
Oxamide	" "	" " "
Gallamide trimethyl ether.....	" "	No reaction.
Urea**.....	Very faint reaction.	Reaction.
Biuret.....	No reaction.	Very faint reaction.
Urethane.....	" "	No reaction.
Allantoin*.....	Distinct reaction.	Reaction (0.003 mg. N).
Asparagine.....		No reaction (α-amino only).

* Neuberg.[3]

** Abderhalden and Schmidt.[6]

CONCLUSIONS.

It is evident from the results of this and the foregoing paper that the ninhydrin reaction is open to serious objections. The appearance of a positive blue color, even if proved spectroscopically to be the ammonium salt of diketohydrindylidene-diketohydrindamine, does not show definitely the presence of amino-acids. Thus, its application to saliva, urine, serum, etc., unless precautions are taken to remove ammonium salts and certain amines, can lead to totally erroneous conclusions. Of the three classes of substances which give a positive test, amino-acids are the most reactive, as they give a strong reaction when in a concentration of 1 cc. = 0.05 mg. nitrogen, whereas the two other classes fail to react in a concentration of 1 cc. = 0.1 mg. nitrogen, so that ilution of the liquid to be tested until its concentration of nitrogen is 0.1 mg. per cc., and the subsequent heating of 1 cc. of such a